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THE UNIVERSITY OF ALBERTA

ENDOTHELIUM-DEPENDENT RELAXATION IN EXPERIMENTAL ATHEROSCLEROSIS IN THE RABBIT

by

© R.L. JAYAKODY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (MEDICINE)

EDMONTON, ALBERTA

FALL 1988

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DEDICATION

I dedicate this thesis to my wife, Lalitha, my two sons, Ruwan and Ranil and to my parents.

ABSTRACT

Atherosclerosis and coronary artery spasm have been associated with abnormalities in the vascular endothelium. The hypothesis that impaired endothelial function is an early marker during atherosclerosis was tested in young rabbits fed a high cholesterol diet. Endothelial function was assessed as endothelium-dependent relaxation (EDR) to acetylcholine. The EDR to acetylcholine was persistently impaired in the aortae of atherosclerotic rabbits, while no difference was seen in the relaxant response to the endothelium-independent vasodilator, sodium nitrite. This finding suggests that relaxation of vascular smooth muscle *per se* is not impaired in the atherosclerotic rabbit aorta. A bioassay for endothelium-dependent relaxation factor (EDRF) suggested that impairment of EDR in atherosclerosis arises from impaired production of EDRF. When the atherogenic diet was replaced by a normal diet, neither regression of atherosclerotic lesions nor restoration of EDR to acetylcholine was seen. These findings provide additional evidence that endothelial function is impaired in atherosclerotic vessels.

When similar studies were carried out using older animals, an apparent dissociation in the extent of atherosclerosis was noted. In the older rabbits, no development of atherosclerosis occurred but the EDR remained impaired. Microscopic evidence of abnormalities of endothelial cells were seen in this group. This finding suggests that impairment in EDR is an early marker of atherosclerosis.

In a further series of experiments, the EDR to acetylcholine was assessed in rabbits made diabetic with alloxan. These animals showed mild hypercholesterolemia, severe hypertriglyceridemia and glycosuria but no evidence of atherosclerosis was seen. The EDR to acetylcholine remained unimpaired. These findings suggest that in the absence of atherosclerosis, diabetes *per se* does not impair EDR to acetylcholine.

Finally, the effects of the calcium channel antagonists, nicardipine and diltiazem, on EDR were assessed. No inhibitory effects were seen. This suggests that calcium channel antagonists may not be influencing vasospasm through effects on EDR.

Thus, there appears to be a close link between atheroma, endothelial function and control of vascular tone.

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INTRODUCTION

Atherosclerosis is a major cause of morbidity and mortality, and together with hypertension, accounts for about 50% of the diseases afflicting the human race (Kannel and Thom, 1986). In humans, the atherosclerotic lesions are seen predominantly in coronary and cerebral arteries and in the abdominal aorta. Morphologically, these lesions appear as localized, raised plaques on the intimal surface. These lesions, on their own or through different complications, cause narrowing of arteries leading to ischaemia in distal areas. The effects of such lesions are seen as clinical syndromes of angina pectoris, myocardial infarction and intermittent claudication.

The major risk factors in the development of atherosclerosis in humans are:

1. hypercholesterolemia
2. hypertension
3. tobacco smoking and
4. diabetes mellitus.

Arterial smooth muscle cells, endothelial cells, platelets and lipoproteins appear to be important in the pathogenesis of atherosclerosis (Titus and Kim, 1985). The initial intimal lesions of atherosclerosis are believed to originate at sites of endothelial injury, even though the form of endothelial injury is not well established (Ross, 1986). The consequent exposure of subendothelial collagen initiates platelet aggregation. Due to the effects of the different peptides (including growth factors) released by the platelets and the permeation of low density lipoproteins (LDL) from the plasma to the subendothelial layer, the smooth muscle cells of the tunica media migrate to the intima and undergo mitosis. The dividing muscle cells in the intima appear to produce the collagen, elastic fibers of the tissue matrix and glycosaminoglycans (Burke and Ross, 1979). Lipids in the plasma, including cholesterol, enter the lesions and accumulate in cellular and extracellular locations leading to

the development of atherosclerosis. Some of these lesions result in elevation of the intimal surface and are seen as plaques.

Among the risk factors listed above, of particular interest to the present investigation is hypercholesterolemia. Different investigators have postulated several mechanisms by which hypercholesterolemia may cause injury to the vascular endothelium.

1. High cholesterol concentrations in the serum may alter the cholesterol: phospholipid ratio of the plasma membranes of endothelial cells and alter their physical properties (Jackson and Gotto, 1976). Such changes could decrease the malleability of the endothelial cells leading to cell retraction as seen over fatty streaks in hypercholesterolemic monkeys (Faggiotto *et al.*, 1984). Such sites may act as foci of entry of lipids into the intima and as sites of aggregation of platelets.
2. Monocytes in the blood stream exposed to chronic hypercholesterolemia have been shown to migrate and penetrate areas of altered endothelium in hypercholesterolemic pigs (Gerrity *et al.*, 1985). Such monocytes may transform into foam cells and participate in the formation of fatty streaks and plaques. Monocytes exposed to normocholesterolemia do not appear to have these properties.
3. Cathcart *et al.*, (1985) observed that low density lipoproteins (LDL) exposed to macrophages, get oxidized, and such oxidized LDL is toxic to cultured fibroblasts. It is postulated that LDL oxidized by macrophages in lesions of atherosclerosis is toxic to the endothelium and could cause endothelial injury (Ross, 1986). In conditions of hypercholesterolemia, the increased cholesterol is manifested as an increase in LDL. Hence, in conditions where the LDL is increased, endothelial injury could occur.

The initial forms of endothelial injury may not be identifiable by light microscopy. However, they may be associated with derangements of endothelial function. An example

of such a derangement of endothelial function is the formation and secretion of growth factors (Ross, 1986).

One of the functions of the vascular endothelium described recently is the production of a substance with vasodilator properties referred to as endothelium-derived relaxation factor (Furchgott and Zawadzki, 1980a; Furchgott, 1983). When isolated rings of arteries (kept in a pre-contracted state) were studied in tissue baths, various agents including acetylcholine, carbachol, thrombin and calcium ionophore A23187 produced a relaxant response (Furchgott, 1983). This relaxant response was abolished by removal of the endothelium. Thus, it was shown that the endothelial cells were obligatory for this relaxation. This relaxant response is referred to as endothelium-dependent relaxation (EDR).

The obligatory role of endothelial cells was explained by the hypothesis that the various agents mediating EDR act on the endothelial cells to release a substance which in turn diffuses onto the medial smooth muscle layer mediating relaxation. This hypothesis was confirmed by transferring the vasodilator substance (endothelium-derived relaxation factor - EDRF) from a donor tissue with endothelium onto a recipient tissue without endothelium and showing relaxation in the latter (Furchgott, 1980a).

The endothelium appears to be important in the interactions of platelets with the vessel wall. Depending on the presence or absence of endothelium, opposite responses have been seen in pre-contracted canine coronary arteries in response to products of platelet aggregation (Cohen *et al.*, 1983a). In the presence of intact endothelium, products of platelet aggregation (principally serotonin and adenosine diphosphate) produced a relaxation response. In the presence of endothelial injury, the products of platelet aggregation elicited a contractile response. Thus, with endothelial injury (as in atherosclerosis), it is possible that the impaired EDR may result in vasospasm in response to products of platelet aggregation. Smooth muscle contraction mediated by these metabolites (vasospasm) may limit the flow of blood to distal areas. These effects will be

marked at sites of vascular narrowing, such as in sites of atherosclerosis in the coronary circulation. Such vasospasms may contribute to angina pectoris and myocardial infarction. It is also possible that the endothelial injury seen in atherosclerosis may be associated with or preceded by impaired production of EDRF. To further elucidate the above, it was decided to investigate the following hypotheses.

1. Atherosclerosis induced by feeding a high cholesterol diet may be accompanied by an impairment of EDR.
2. Any impairment of EDR in experimental atherosclerosis may be due to failure of production of EDRF rather than due to impaired diffusion of EDRF or impaired relaxation of the vascular smooth muscle.
3. Impairment of the EDR may precede the development of atherosclerosis and serve as a marker of the functional abnormality in the endothelium that allows the "leakage" of lipids from the blood stream to the intima.
4. The susceptibility to atherosclerosis/impairment of EDR may vary as a function of the age of the animals.
5. Reversal to a normal diet after an initial period of feeding a high cholesterol diet may lead to regression of the atherosclerotic process and restoration of EDR.

These hypotheses were tested on the rabbit model of experimental cholesterol atherosclerosis.

As there is a link between atherosclerosis and diabetes mellitus (Beach and Strandness, 1980) and since there are endothelial cell abnormalities in diabetes also (Bern and Busick, 1985), the following hypothesis was tested in rabbits rendered diabetic by injecting alloxan.

6. A diabetic state in animals may lead to an impairment of EDR as diabetes in humans is known to be a risk factor for the development of atherosclerosis.

Administration of calcium channel blockers have been reported to be beneficial in the treatment of coronary artery spasms (Pepine *et al.*, 1983). Therefore, the following

hypothesis relating calcium channel blockers and EDR was tested in aortae of normal rabbits.

7. Calcium channel blockers may not lead to an impairment of EDR as these drugs are known to be beneficial in atherosclerotic coronary artery disease in humans.

In the rabbit model of atherosclerosis, endothelial involvement is a consistent feature (Klimov *et al.*, 1981). Hence as a model of endothelial cell injury, this model appeared to be particularly suitable. In addition, for an insidious disease like atherosclerosis, lesions can be produced over relatively short periods of time and these lesions have been well described previously (Duff *et al.*, 1957; Prior *et al.*, 1961; Imai *et al.*, 1966). Compared to pigs and monkeys, the rabbit model has the additional advantages of being plentiful, easily handled, relatively inexpensive and biologically well characterized (Clarkson *et al.*, 1974, Jokinen *et al.*, 1985).

The thoracic aortae obtained from atherosclerotic rabbits were used in this investigation for the following reasons.

1. In the rabbit model, the thoracic aorta shows the greatest involvement with lesions of atherosclerosis.
2. Rabbit aorta has been morphologically and pharmacologically well characterized (Wolinsky and Glagov, 1964; Fleisch, 1974; Besse and Furchgott, 1976).
3. Being a vessel of considerable diameter, experimental manipulations were possible without damaging the endothelium.
4. Endothelium-dependent relaxations to several pharmacological agents (e.g., acetylcholine, thrombin, calcium ionophore and substance P) have been demonstrated in the rabbit aorta (Furchgott, 1983).

For induction of atherosclerosis, a diet containing 2% cholesterol and high content of lipids (24%) was used. The lipids of this diet were high in saturated and monounsaturated fatty acids. Diets with these features (i.e., high in cholesterol and saturated fatty acids) have been found to be atherogenic in humans.

LITERATURE REVIEW

ENDOTHELIUM-DEPENDENT RELAXATION

Furchgott and Zawadzki (1980a) demonstrated that acetylcholine produced relaxation in rings of rabbit aorta that had been pre-contracted with noradrenaline. This relaxation to acetylcholine was strictly dependent on the presence of endothelial cells. These same authors showed that endothelium-dependent relaxation (EDR) was mediated by a factor which is produced in the endothelial cells, which diffuses onto the smooth muscle to cause relaxation. This factor is referred to as endothelium-derived relaxation factor (EDRF).

The initial experiments reported on EDR were designed with a view of getting insights into the nature of EDRF. The range of concentrations over which acetylcholine produced relaxation in aortae pre-contracted with noradrenaline was from 0.01 to 3.0 µmol/l. At concentrations in excess of 3.0 µmol/l of acetylcholine, a contractile response was produced. The EDR seen at lower concentrations of acetylcholine and the contractile response seen at higher concentrations of acetylcholine were both blocked by atropine (Furchgott *et al.*, 1981). Using selective muscarinic receptor ligands, it was shown that EDR to acetylcholine was mediated via muscarinic receptors. Compared to acetylcholine, other cholinergic muscarinic receptor agonists such as methacholine and carbachol were found to be less potent in causing EDR.

Factors affecting endothelium-dependent relaxation

A number of factors have been shown to influence EDR to acetylcholine.

(A) Anoxia - It has been reported that anoxic conditions inhibited the EDR to acetylcholine in canine femoral arteries and in the rabbit aorta (De Mey and Vanhoutte, 1978; Furchgott, 1983). However, the relaxations produced by the endothelium-independent vasodilators like sodium nitrite were not inhibited by anoxia.

(B) Quinacrine (Mepacrine) - It was initially believed that EDRF could be a metabolite derived from membrane phospholipids released through the action of phospholipase A_2.

Quinacrine has been shown to be an inhibitor of phospholipase A_2 (Flower and Blackwell, 1976). When quinacrine was added prior to or after the addition of acetylcholine, it was observed that it inhibited the EDR (Singer and Peach, 1983). This inhibition appeared selective for the EDR since quinacrine failed to inhibit the relaxation produced by isoproterenol and glyceryl trinitrate.

(C) Cyclooxygenase inhibitors - Aspirin (100 mmol/l) and indomethacin (40 mmol/l) were found to have no inhibitory effect on the EDR to acetylcholine (Furchgott, 1983). Hence cyclooxygenase products of arachidonic acid metabolism were excluded as mediators of EDR.

(D) 5, 8, 11, 14 - eicosatetraynoic acid (ETYA) - This triple bond analogue of arachidonic acid has been used as a combined cyclo- and lipooxygenase inhibitor (Flower, 1974). When ETYA was added during the course of an acetylcholine induced relaxation of rabbit aorta that was pre-contracted with noradrenaline, it rapidly antagonized the EDR (Furchgott and Zawadzki, 1980a). The action of ETYA was also found to be selective for EDR as it did not antagonize the relaxant responses produced by isoproterenol and sodium nitrite.

(E) Nordihydroguaiaretic acid (NDGA) - This agent is known to be both an antioxidant and an inhibitor of lipooxygenase. NDGA was reported to be a very effective inhibitor of endothelium-dependent relaxation (Furchgott, 1983).

(F) Hydroquinone - It was initially thought that EDRF may be a free radical (Furchgott *et al.*, 1981). Results with most of the agents tested were either negative or equivocal. However, hydroquinone with its free radical scavenger properties consistently inhibited EDR.

Nature of EDRF

The inhibition of EDR by anoxia, atropine, ETYA and quinacrine suggested that acetylcholine, acting on the muscarinic receptors of the endothelial cells, somehow activates a reaction sequence in which arachidonic acid (or some other unsaturated fatty acid) is liberated from membrane phosphatides. The unsaturated fatty acid liberated is then

oxidized by lipooxygenase in the endothelial cells to a product (EDRF) which diffuses onto the smooth muscle, mediating the relaxation (Furchgott, 1983). As cyclooxygenase inhibitors like aspirin and indomethacin were without effect on EDR to acetylcholine, prostaglandins and prostacyclins were excluded as candidates of EDRF. It was speculated that the factor might be a labile hydroperoxide or a free radical intermediate product. However, all of these findings are not sufficient evidence for a firm conclusion that EDRF is an oxidation product formed via a lipooxygenase pathway. It may be possible that the agents listed above could be inhibiting acetylcholine induced relaxation by some other mechanism. For instance, the lipooxygenase inhibitor BW 755C failed to inhibit acetylcholine induced EDR of rabbit aorta. Also, in addition to the cyclo- and lipooxygenase pathways, a third pathway involving cytochrome P450 is thought to be involved in the metabolism of arachidonic acid (Vanhoutte *et al.*, 1986). Presently, the metabolites of this pathway are not well characterized.

Other agents producing EDR of arteries

(A) Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) - Both these agents have been reported to produce EDR. This was demonstrated in the rabbit aorta and dog femoral artery (Furchgott and Zawadzki, 1980b; De Mey and Vanhoutte, 1981). However, the relaxations to adenosine monophosphate (AMP) and adenosine did not appear to be dependent on an intact endothelium.

(B) Bradykinin - Bradykinin causes strong EDR in canine arteries isolated from several regions (e.g., superior mesenteric, renal, coeliac and splenic) (Cherry *et al.* 1982). In contrast, the relaxation by bradykinin of superior mesenteric arteries of the cat and rabbit are not completely dependent on the presence of endothelial cells. Moreover, the EDR observed in the canine arteries was not inhibited by cyclooxygenase inhibitors, whereas relaxations by bradykinin of cat and rabbit arteries (with and without intact endothelial cells) were completely inhibited by cyclooxygenase inhibitors. These results indicate that

there could be differences in the endothelium-dependent relaxant substances liberated from the vascular beds of different animal species (Fostermann *et al.*, 1985).

(C) Substance P - Substance P relaxes isolated arteries from rabbits, dogs and cats which are strictly dependent on the presence of endothelial cells (Zawadzki *et al.*, 1981). However, compared to acetylcholine, substance P produces a smaller maximal relaxation on the rabbit aorta. This may be partly because desensitization to substance P occurs during the course of exposure.

(D) Histamine - In pre-contracted rings of rat thoracic aorta, histamine produces concentration-dependent relaxation. This relaxation is endothelium-dependent and is thought to be mediated via H_1 receptors (Van de Voorde and Leusen, 1982).

(E) Arachidonic acid - Arachidonic acid, at concentrations of 10-100 µmol/l, produces moderate relaxation of noradrenaline-induced contractions in the rabbit aorta (Singer and Peach, 1983). This relaxation was shown to be dependent on the presence of endothelial cells and was potentiated by indomethacin. However, in dog femoral arteries, it was found that indomethacin (100 µmol/l) completely inhibited the EDR by arachidonic acid. Metabolites formed from isolated dog femoral arteries showed high concentrations of 6-keto PFG_1 levels in preparations where the endothelium was intact. It is known that prostacyclin is spontaneously converted to 6-keto PGF_1. This product was almost completely eliminated by either removing the endothelium or by pretreating the preparation with indomethacin. Based on these results De Mey and co-workers concluded that relaxation of dog femoral arteries by arachidonic acid is mediated by prostacyclin (De Mey *et al.*, 1982). Prostacyclin is believed to be produced in endothelial cells from the added arachidonic acid. Addition of unsaturated fatty acids have been shown by other investigators to increase the fluidity of cell membranes and enhance the rate of certain enzymatic reactions in membranes (Orly and Schramm, 1975; Rimon *et al.* 1978). Hence, it has been proposed that EDR by these unsaturated fatty acids may be the result of an

increase in fluidity of the endothelial cell membrane which facilitates reactions leading to formation of EDRF.

As several pharmacological agents working through different receptor systems are able to elicit EDR, all these agents may be linked to a common pathway leading to the generation of EDRF.

Role of calcium in the release of EDRF

Like acetylcholine, the calcium ionophore A23187 brings about relaxation of isolated rabbit aortic rings by an endothelium-dependent mechanism. This agent is about 10-30 times more potent than acetylcholine in producing relaxation (Zawadzki *et al.*, 1980). The maximum relaxation by A23187 (0.1 µmol/l) had been found to be always greater than that produced by acetylcholine (1-3 µmol/l). It is believed that an increase in the calcium ions in the region of some key Ca^{++} activated enzyme (perhaps a phospholipase) might be an early step in the sequence of reactions mediating the release of EDRF by acetylcholine and A23187 (Furchgott, 1983). The compound A23187 (acting as an ionophore) and acetylcholine (by opening up calcium channels coupled to muscarinic receptors) might facilitate Ca^{++} influx into the region of this enzyme.

Further evidence for the critical role of calcium ions has come from a study by Singer and Peach (1982). They found that eliminating Ca^{++} from the incubation medium inhibited the maximum methacholine induced relaxations by 67% and A23187 induced relaxations by 92%. They also found that the calcium channel blockers, verapamil and nifedipine inhibited maximum methacholine and A23187 induced relaxations by about 40-45%. These experiments suggest that Ca ions play an important role during EDR.

Mechanism of relaxation by EDRF

It has been reported that in certain smooth muscle preparations, there is a positive relationship between increases in cyclic guanosine monophosphate (cGMP) and relaxation (Katsuki and Murad, 1977; Bohme *et al.* 1978). Guanylate cyclase was markedly stimulated by hydroperoxides of arachidonic acid and by free radicals, particularly nitric

oxide and hydroxyl radical (Hidaka and Asano, 1977). Rapoport and Murad (1983) recently obtained results in accord with the speculation that EDRF causes an increase in vascular smooth muscle cGMP. They hypothesized that EDR by acetylcholine as well as endothelium-independent relaxation by nitrovasodilators was mediated through cyclic GMP-dependent protein phosphorylation and dephosphorylation of myosin light chains.

Nitric oxide and EDRF

Nitrovasodilators, which may act by releasing nitric oxide (NO), mimic the effects of EDRF and it has been suggested that EDRF could be nitric oxide (Furchgott *et al.*, 1987; Khan and Furchgott, 1987). Acidified solutions of sodium nitrite (approximate pH 2.0) produce transient relaxations in pre-contracted aortic rings and these relaxations are much larger than the monophasic relaxations produced by neutral solutions of sodium nitrite (0.1 - 10.0 μmol/l). This relaxation is considered to be due to nitric oxide generated from nitrous acid. Transient relaxations have been elicited by dilute anaerobic solutions of nitric oxide also. Inhibitors of EDR to acetylcholine like haemoglobin, hydroquinone and phenidone have been shown to inhibit relaxations elicited by nitric oxide. Generation of O_2 has been shown to hasten inactivations of EDRF while superoxide dismutase prolongs the action of EDRF by protecting it from inactivations. These results have been sited to propose that EDRF is nitric oxide. In a recent study, Palmer *et al.* (1987) assayed the release of EDRF and nitric oxide as the chemiluminescent product of its reaction with ozone. They reported that nitric oxide released from endothelial cells was indistinguishable from EDRF in terms of biological activity, stability and susceptibility to an inhibitor (haemoglobin) and to a potentiator (superoxide dismutase). They reached the conclusion that EDRF could be nitric oxide.

EDR and substances involved in haemostasis

Although vascular endothelium does not encounter acetylcholine *in vivo*, the pharmacological experiments with acetylcholine have indicated the potential importance of the endothelium in vascular spasm. Several substances generated during the process of

haemostasis can cause endothelium-dependent relaxations. Thrombin generated during the coagulation cascade has been shown to cause EDR in several blood vessels including coronary arteries (De Mey *et al.*, 1982; Ku, 1982). The thrombin-induced relaxations can be prevented by heparin.

Several vascular preparations (including canine coronary arteries) showed a contractile response when exposed to products released during platelet aggregation (Cohen *et al.*, 1983 a, 1983 b). Some of the effects mediated by platelets are attributed to serotonin and ADP. In canine coronary artery rings, such contractions are greater in the absence of endothelium. Endothelium-dependent relaxations have been elicited by canine and human platelets in pre-contracted artery rings with endothelium. However, endothelium denuded rings showed a contractile response under similar experimental conditions. Thus, substances involved in haemostasis (e.g., thrombin) and products of platelet aggregation (e.g. serotonin, ADP) appear to influence coronary artery tone (Cohen and Vanhoutte, 1985). The vasodilator effects of these agents appear to depend upon an intact endothelium. Endothelium dysfunction or damage as seen in atherosclerosis could lead to a reversal of the vasodilator responses and thus promote the development of arterial spasm (Heistad *et al.*, 1984; Vanhoutte *et al.*,1986).

Coronary artery spasm and endothelial function

Atherosclerosis affecting the coronary arteries is an important cause of angina pectoris. This symptom is commonly due to a fixed lesion (narrowing) of one of the coronary arteries. However, in many cases, no fixed lesions are observed at arteriography and it has been shown that in the early stages of atherosclerotic disease, spasm may be a critical cause of angina pectoris. Several mechanisms have been postulated as causing coronary vasospasm.

(1) Asymmetric activation of sympathetic nerves to the heart leading to α-adrenoceptor mediated vasoconstriction (Yasue *et al.*, 1976).

(2) Incorporation of cholesterol into membranes of smooth muscle cells, rendering the vessels more susceptible to activation by autonomic nerves (Marx, 1980).

(3) Decrease in number and affinity of α-adrenoceptors located on sympathetic nerves (Weiss and Smith, 1983).

(4) Platelet aggregation and release of vasoactive substances (Sobel *et al.*, 1981).

Both β_1 and α_1 adrenoceptors have been demonstrated in canine coronary arteries. Both these receptors are acted upon by the released noradrenaline. Under normal conditions, these vessels relax due to the dominance of the β_1 receptors. However, in the presence of β receptor antagonists, a vasoconstrictor response may be produced (Robertson *et al.*,1982). The coronary arteries have been shown to have a cholinergic innervation also (Feigl, 1969). Acetylcholine released from these terminals acts on the muscarinic receptors on the sympathetic nerve terminals to reduce the output of noradrenaline, and thereby reduce the relaxation mediated by β_1 receptors. Thus, muscarinic agonists can precipitate coronary artery spasm (Endo *et al.*, 1976). If the smooth muscle of the coronary arteries becomes hypoxic, its responsiveness to β adrenoceptor stimulation is lost and constrictor responses are exaggerated (Van Neuten and Vanhoutte, 1980).

The healthy endothelial lining of the coronary artery plays an important role in preventing coronary artery spasm. Acetylcholine, ADP, ATP, bradykinin, noradrenaline, serotonin and thrombin could all initiate EDR in coronary arteries (Cocks and Angus, 1983; Vanhoutte and Miller, 1985). In addition, the vascular endothelium produces the vasodilator, prostacyclin. Hence, with a healthy intact endothelium the effect of all the above agents is a relaxant response.

At sites of endothelial disease and injury (e.g., over atherosclerotic plaques), the generation of prostacyclin is decreased (Sobel *et al.*, 1981). Platelet aggregation leads to release of the vasoconstrictor thromboxane A_2. In the absence of endothelium, the effect of

all the agents mentioned above (noradrenaline, serotonin and thrombin) is a contractile response. Such a situation will promote vasospasm.

In isolated human epicardial coronary artery rings, the presence of atherosclerosis has been shown to potentiate the contractile responses to histamine (Ginsburg *et al.*, 1984). Both histamine and serotonin have been shown to provoke coronary artery spasm in miniature swine at sites where the endothelium is denuded (Shimokawa *et al.* 1983). Patients with spontaneous coronary artery spasm have been shown to have atherosclerotic lesions at sites of spasm (Dalen *et al.*, 1982). These examples highlight the important role of the endothelium in regulating coronary artery tone. At sites of endothelial injury (e.g., lesions of atherosclerosis), platelet aggregation and vasoconstriction occurs. Subsequently, there is formation of thrombin and blood clot. As the blood supply to the myocardium is reduced, hypoxia and further arterial constriction occurs. Thus, the endothelium-dependent relaxant responses appear to be very important in preventing coronary vasospasm (Shepherd and Vanhoutte, 1985).

Endothelium-dependent contractions

In addition to endothelium-dependent relaxations, endothelium-dependent contractions have been described also (De Mey and Vanhoutte, 1982). These are thought to be mediated by more than one factor (Hickey *et al.*, 1985). In comparison with EDR, endothelium-dependent contractions have been seen in a fewer number of blood vessels (Vanhoutte, 1987 a). The phenomenon of endothelium-dependent contraction has been reported in canine pulmonary veins (to arachidonic acid), aortae of spontaneously hypertensive rats (to serotonin) and canine coronary arteries (to anoxia).

CHOLESTEROL METABOLISM

The items of food in our diet can be divided into carbohydrates, lipids and proteins. The important components in dietary lipids include triglycerides, phospholipid and cholesterol. The average consumption of lipids by an individual on a typical American diet is about 150 g (Rizek *et al.*, 1974). Among the lipids are a group of substances called

sterols. These have a molecular structure called the perhydrocyclopentanophenanthrene ring, which is made up of three 6 carbon rings and one 5 carbon ring. Natural compounds with alcohol groups attached to this ring structure are called sterols. In the diet, the sterols come from both animal and plant sources. The important sterol from animal food is cholesterol. Eggs, meat, milk and butter are rich sources of cholesterol. The cholesterol can exist as free and esterified forms. While free cholesterol has a limited ability to interact with water, the esterified cholesterol does not interact with water at all.

As most dietary lipids are insoluble in water, the solubility of these lipids has to be increased prior to absorption. The digestion and absorption of lipids can be discussed under the following headings (Shiau, 1987).

1. Intraluminal digestion
2. Micellar solubilization
3. Permeation from lumen to cell
4. Intracellular reesterification
5. Chylomicron formation and secretion

1. Intraluminal digestion - The triglycerides are acted upon by pancreatic and intestinal enzymes and broken down to free fatty acid, monoglycerides and glycerol. The phospholipids are broken down to lysophospholipids by phospholipase. Cholesterol esters are hydrolysed by pancreatic cholesterol esterase.

2. Micellar solubilization- The products of exocrine enzyme action go through a process of micellar solubilization (Hofmann and Small, 1967). This process involves mixing with bile salts. In man, these salts are primarily cholate and deoxycholate conjugated with glycine or taurine. Bile salts have hydrophilic and hydrophobic groups. In the intestinal lumen, the bile salts can form highly charged aggregates called micelles (Carey and Small, 1972). Conversion of water insoluble lipids into soluble micellar aggregates is referred to as micellar solubilization. As the products of intraluminal digestion are insoluble in water, they all go through micellar solubilization prior to absorption.

3. Permeation from lumen to cell - The digested food and water in the lumen of the intestine is thought to exist in two phases. These are referred to as bulk phase and unstirred water layer. Bulk phase refers to food and water in the central areas of the lumen which is propelled down by peristalsis. The water adjacent to the luminal lining is not in equilibrium with the bulk phase and is referred to as the unstirred water layer. This layer is sometimes referred to as mucous barrier or microclimate also. Micelles transport products of lipid digestion to the absorption site (i.e., intestinal epithelial cell membrane). The intracellular fatty acid uptake depends on fatty acid concentration in the unstirred water layer (Thomson, 1980). In addition, the permeability coefficient of the membrane influences entry of fatty acids. The fatty acids dissociate from the bile salt micelle. The uptake into the enterocyte (intestinal epithelial cell) appears to be a process of passive absorption which is concentration dependent. The phospholipids are absorbed as lysophospholipids.

The cholesterol in the intestinal lumen could come from three sources.

1. Cholesterol in ingested food.
2. Biliary cholesterol.
3. Cholesterol in desquamated enterocytes.

The latter two sources could account for as much as 50% of the cholesterol in the lumen. Cholesterol is found in free and esterified forms (Treadwell and Vahouny, 1968). For cholesterol esters to be absorbed, they must be hydrolyzed to free cholesterol. This hydrolysis is catalyzed by pancreatic cholesterol esterase. Cholesterol is absorbed passively as a monomer and dissociation of cholesterol from micelles must occur before absorption.

4. Intracellular reesterification - The long chain fatty acids absorbed into the enterocyte bind with a fatty acid binding protein. This complex of the fatty acid and binding protein gets transported to the endoplasmic reticulum for reesterification to triglycerides. Medium

chain fatty acids do not bind with the binding protein, but get absorbed into the portal vein without reesterification.

The reesterification of fatty acids in the enterocyte can occur predominantly via two pathways (Johnston, 1978).

A. Monoglyceride pathway - The fatty acids interact with coenzyme A to form acyl coenzyme A. This combines with monoglycerides to give diglycerides. The diglycerides recombine with acyl coenzyme A to give rise to triglycerides. This reesterification pathway appears to be the major pathway for triglyceride synthesis during absorption of lipids.

B. Phosphatidic acid pathway - Two molecules of acyl coenzyme A combine with α-glycerophosphate to give rise to phosphatidic acid. This acid can be used for synthesis of triglycerides and phospholipids. This pathway is linked with carbohydrate metabolism and appears to play an important role during fasting.

The enterocytes use the fatty acids for different functions. As described, a part of it is used for the synthesis of triglycerides. Some fatty acids become oxidized in the enterocyte. The enterocyte has also been shown to have the capacity for *de novo* synthesis of fatty acids from acetate. This pathway of fatty acid synthesis does not appear to be under hormonal control (Shakir *et al.*, 1978).

The lysophospholipids are reesterified in the enterocyte to phospholipids. The cholesterol in the enterocyte could come from three sources. These are,

1. the cholesterol absorbed from intestinal lumen
2. the cholesterol filtered from plasma LDL and HDL and
3. *de novo* synthesis from acyl coenzyme A.

As 80-85% of cholesterol found in the lymph is in the esterified form, the cholesterol in the enterocyte should also get esterified prior to absorption.

5. Chylomicron formation and secretion - The lipid carrying particle called chylomicron is formed in the enterocyte. The reesterified fatty acids (triglycerides), phospholipids, free and esterified cholesterol and a special class of proteins called apolipoproteins are used for

the synthesis of chylomicrons. Analysis of mesenteric lymph during fat absorption shows large amounts of chylomicrons.

Chylomicron - This is a spherical particle of 750-6000 Å diameter. The hydrated density (g/ml) is less than 0.95. The composition of this particle is as follows: triglycerides 78%, cholesterol 10.5%, proteins 4% and phospholipids 7.5% (Sabine, 1977). The core of the particle contains triglycerides and esterified cholesterol. The surface has a phospholipid monolayer, some free cholesterol and the apolipoproteins. Over 75% of this particle is formed of triglycerides. When the intestinal lipid load is increased, the number and size of the chylomicrons are known to increase.

The intestinal mucosa has been shown to produce VLDL also. The amount of VLDL produced is small and this fraction is known to increase during fasting (Ockner *et al*, 1969).

Apolipoprotein synthesis in intestine - Although only small amounts of these proteins are found in the chylomicrons, they appear to play an important role. These apolipoproteins function as receptors and are recognized at specific sites (e.g., vascular endothelium, liver). Apolipoproteins, along with phospholipids, appear to be important in the release of lipoproteins from the enterocyte also. Apolipoprotein A and B are synthesized in the small intestine. However, the majority of apolipoproteins in the mesenteric lymph appear to be derived from circulating lipoprotein particles or their remnants (Schaefer *et al*., 1978).

When the chylomicron particle leaves the enterocyte, it has been shown to have apolipoproteins A and B. After entering the lymph, this chylomicron particle has been shown to acquire apolipoprotein E and C. The apolipoprotein C of the chylomicron is involved in the activation of the enzyme lipoprotein lipase. The triglycerides in the chylomicrons are hydrolyzed by this enzyme, especially in capillaries of muscle and adipose tissue, and the particle becomes smaller in size and loses some apolipoprotein also. This chylomicron remnant is recognized and cleared by the liver. Chylomicrons are the most important particles carrying absorbed lipid out of the bowel. In this way, much of

dietary and biliary cholesterol and the component synthesized in the intestine is delivered directly to the liver.

Before the transport and metabolism of lipids and cholesterol is discussed, an introduction of the different lipoprotein particles will be helpful.

Very low density lipoprotein (VLDL) - This is a spherical particle of 300-700 Å diameter. The density is about 0.98 - 1.03 g/ml. The composition of this particle is as follows: Triglycerides 55%, cholesterol 17%, proteins 8% and phospholipids 19% (Sabine, 1977). This particle is synthesized primarily in the liver and to a lesser extent in the intestine. Constitutionally and functionally, VLDL can be regarded as small chylomicrons. Compared to chylomicrons, the half life of this particle in plasma is much longer (6-12 hours). The primary function of VLDL is to transport triglycerides away from the liver.

Low density lipoprotein (LDL) - This is a spherical particle of 150 - 300 Å diameter. The density range is about 1.03 - 1.09 g/ml. The composition of this particle is as follows: triglycerides 10%, cholesterol 41%, proteins 21% and phospholipids 28%. LDL is the most abundant lipoprotein class in humans. As it is the principal cholesterol carrying particle, abnormally elevated LDL has been associated with atherosclerosis.

High density lipoprotein (HDL) - This is an elongated or disc shaped particle, 90 - 350 Å in size. The density range is about 1.1 - 1.2 g/ml. The composition of this particle is as follows: triglycerides 6%, cholesterol 11%, proteins 58% and phospholipids 25%. Apolipoprotein E of HDL has been identified as a cofactor for the enzyme lipoprotein lipase.

The enzymes lipoprotein lipase (LPL) and lecithin:cholesterol acyl transferase (LCAT) also play an important role in lipid metabolism. Their functions will be considered briefly.

Lipoprotein lipase (LPL)

This enzyme is found in the walls of capillaries. It is anchored to the surface by proteoglycan chains of heparan sulphate. Under normal conditions, significant amounts of

this enzyme are not found in the circulation. However, injection of heparin leads to release of this enzyme and clearing of lipids from plasma (Mayes, 1988). Both phospholipid and apolipoprotein C appear to be cofactors for this enzyme. Hence, both chylomicrons and VLDL provide the cofactor and the substrate. Hydrolysis of lipids occurs while the enzyme is still attached to the capillary endothelium. After the action of LPL, the chylomicrons lose approximately 90% of their triglycerides and apolipoprotein C.

Lecithin:cholesterol acyl transferase (LCAT)

This enzyme is synthesized in the liver and has the function of esterification of cholesterol in the plasma. The normal substrates of this enzyme appear to be HDL or unesterified cholesterol. Both these substrates are present in the circulation. The combination of lecithin and cholesterol is catalysed by this enzyme leading to the formation of lysolecithin and cholesterol esters (Sabine, 1977). The resulting cholesterol esters are transferred to other lipoproteins. LCAT participates in transport of cholesterol from the periphery to the liver also.

Lipoprotein transport

The transport of lipoprotein occurs via two pathways.

1. Exogenous pathway
2. Endogenous pathway

A schematic representation of these two pathways is shown in Figure 1.

1. Exogenous pathway- This pathway transports exogenous lipids that enter the circulation from the intestine. The chylomicrons secreted into the lymph subsequently enter the blood stream. Triglycerides are hydrolysed by the action of lipoprotein lipase and the fatty acids removed. The remaining particle (chylomicron remnant) is taken into the hepatocytes by a process called receptor mediated endocytosis. Inside the liver cells the remnant is broken down to release free cholesterol. This cholesterol can be either stored in hepatocytes as cholesterol esters, excreted into bile or used to form endogenous lipoproteins.

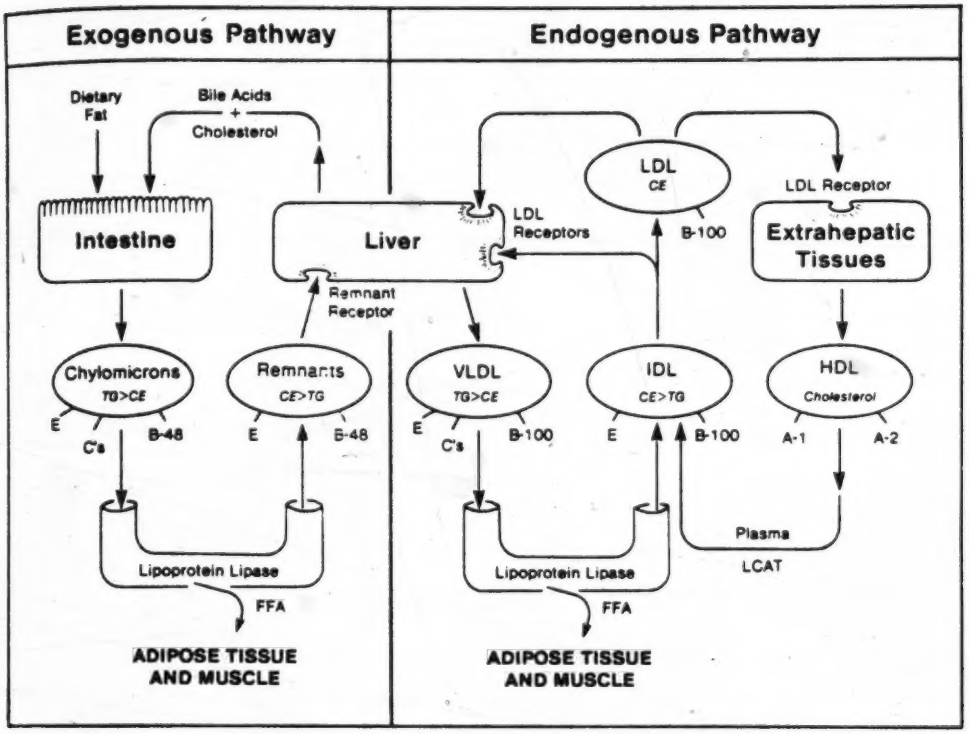

Figure 1. Model for the metabolism of plasma lipoproteins, showing the separate pathways for transport of endogenous and exogenous lipids.
CE denotes cholesterol esters; FFA, free fatty acids; TG, triglycerides; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.
A-1, A-2, B-48, B-100, C's and E represent the apoproteins associated with the indicated lipoprotein particle (Reproduced with permission from author and publisher, Brown MS and Goldstein JL, Goodman and Gilman's *The Pharmacological Basis of Therapeutics, ed. 7*, 1985).

2. Endogenous pathway- The cholesterol and triglycerides rich particle called VLDL is released into the plasma from the liver. The triglycerides are removed from VLDL by the action of lipoprotein lipase and through an intermediate density lipoprotein stage becomes LDL. The LDL particle is rapidly cleared by the liver by a process of receptor mediated endocytosis. The LDL not taken up by the liver circulates in the blood. These particles are eventually degraded by binding to LDL receptors in the liver and extrahepatic tissues.

The circulating LDL acts as the major source of cholesterol in the plasma. When liver and other tissues require cholesterol, they synthesize LDL receptors and cholesterol is taken into the cell (Brown and Goldstein, 1985 a, 1985 b). When cholesterol is not needed by the tissues, the LDL receptor synthesis is decreased.

In addition to the specific LDL receptors, the LDL is also taken up by other pathways which are seen in macrophages and other scavenger cells. When the plasma cholesterol concentration increases, the degradation by these pathways increases also. This may contribute to deposition of cholesterol in arterial walls leading to atherosclerosis.

LDL metabolism

Triglycerides and cholesterol are transported out of the liver by VLDL. The VLDL can be metabolized in two ways. It can be metabolized through an intermediate density lipoprotein (IDL) stage to LDL (this pathway is thought to predominate in man). In addition, after the action of lipoprotein lipases and removal of triglycerides, the remnant is taken up by the liver (this pathway is thought to predominate in the rat). Hence, a variable proportion of the cholesterol derived from the hepatic sterol pool and incorporated into VLDL, finally end up circulating in the plasma LDL fraction.

LDL can be internalized by receptor-dependent or receptor-independent mechanisms (Simionescu and Simionescu, 1986). Receptor-dependent LDL uptake is seen in organs like the liver and endocrine glands which manifest high rates of LDL uptake. In many animal species,the liver is thought to account for the uptake of 50 - 75% of all LDL cleared from the plasma. Using methylated - LDL, it has been shown that receptor-independent

LDL uptake occurs in all organs in the body (Spady *et al.*, 1983; Spady *et al.*, 1985). In the enterocyte,the LDL uptake occurs through both pathways, each contributing to about 50% of entry. In most tissues with low LDL uptake, the entry occurs through receptor-independent processes.

When LDL concentration is changed, the relative importance of receptor-dependent and receptor-independent uptake in each organ is known to change. However, LDL cholesterol taken up by either of these two processes has been shown to have a similar suppressive effect on hepatic cholesterol synthesis. When there is overproduction of LDL or when LDL receptor activity is reduced, the receptor-independent system appears to play a greater role in LDL removal from plasma (Spady *et al.*, 1983). In these situations, cholesterol balance seems to be achieved by marked elevation of plasma LDL-cholesterol.

Receptor activity can also be suppressed by feeding cholesterol. Thus, whether a manipulation alters hepatic LDL clearance (and circulating LDL cholesterol) critically depends on the capacity of that particular animal to compensate for the manoeuvre by changing the hepatic cholesterol synthesis. It has been shown that at a normal plasma LDL concentration, receptor-dependent LDL cholesterol uptake accounts for approximately 62% of total LDL cholesterol degradation. With a loss of LDL receptor activity (types of familial hypercholesterolemia), there is an associated increased LDL production rate leading to elevated serum LDL-cholesterol concentration. The receptor numbers in patients with familial hypercholesterolemia have been shown to be approximately 48% of normal in the heterozygous group and approximately 4% in the homogenous group (Goldstein and Brown, 1983).

Function of HDL

HDL serves as a cholesterol acceptor in the periphery. Because of this function of reverse cholesterol transport, high concentrations of HDL are negatively correlated with ischaemic heart disease.

Crossing of plasma macromolecules through the endothelial barrier

The transport of molecules across the endothelium is governed by three groups of factors.

1. Plasma driving forces (hydrostatic and osmotic pressures).
2. Physicochemical properties of the permeant molecules (size, shape, charge and concentration).
3. Surface properties and specific activities of the endothelial cells.

Endothelium is known to have the basic properties of a polarized epithelium and is highly specialized for transport. Endothelial participation in the blood tissue exchange appears to be a refined and dynamic phenomenon (Steinberg *et al.*, 1985; Simionescu *et al*, 1987). Entry of macromolecules into the endothelial cells can occur through processes of endocytosis and transcytosis. The process where cells are continuously ingesting bits of their plasma membrane in the form of small vesicles is called endocytosis. The process where plasma molecules are transported across the cell to the interstitial fluid is referred to as transcytosis. Both these processes could occur after adsorption of the particles to the surface (adsorptive) or without adsorption to the surface (bulk phase). Adsorption can be either non-specific (e.g., electrostatic binding) or specific (e.g., receptors) in nature. The internalized molecules can reach the lysosomal compartment (endocytosis) or can be transported across the cell to the interstitial fluid (transcytosis).

Endocytosis can occur either by clathrin-coated pits and coated vesicles (adsorptive and receptor-mediated endocytosis) or by fraction of plasmalemmal vesicles (fluid-phase endocytosis or pinocytosis). Transcytosis can be carried out by plasmalemmal vesicles shuttling between the two endothelial surfaces or by transient transendothelial channels. Transcytosis can occur either in the fluid phase or after binding to a surface receptor (receptor-mediated transcytosis).

It has been demonstrated that arterial endothelium is able to take up LDL by saturable high affinity receptors located in coated pits and by the low affinity receptors confined to plasmalemmal vesicles. Animal cells that need cholesterol make the LDL

receptors and insert them into the plasma membrane. Most of these receptor proteins associate spontaneously with the coated pits and those that do not are induced to migrate to coated pits by the binding of LDL (Alberts *et al.*, 1983). Since coated pits are constantly pinched off to form coated vesicles, all of the LDL particles that bind to LDL receptors are rapidly internalized. The coated vesicles lose their coat and fuse with other vesicles to form larger vesicles called endosomes. These fuse later with the lysosomes. Within 10-15 minutes of binding to the cell surface receptors, LDL is delivered to lysosomes where cholesterol esters are hydrolyzed to free cholesterol and thereby becomes available to the cell for new membrane synthesis. If excess cholesterol accumulates in the cell, it shuts off both the cell's own cholesterol synthesis and its synthesis of LDL receptor proteins so that less cholesterol is made and less is taken up by the cell.

Vesicles originating from the luminal surface have been shown to fuse with internal vesicles, which had in turn fused with vesicles opening to the abluminal surface (Palade *et al.*, 1979). This leads to the formation of a transendothelial channel. These temporary through-and-through channels might be a mechanism for facilitating movement of macromolecules through the endothelium. How frequently these channels are formed and how long they persist are still uncertain. These gaps could allow non-specific transport of LDL. Whether or not these channels play an important role, non-specific fluid endocytosis and rapid movement of endocytic vesicles across the narrow cytoplasmic band of the capillary endothelium could, in theory, account for much or all of LDL transport. However, most of these studies were carried out in capillary endothelial cells and whether these conclusions apply equally to endothelial cells of large arteries, needs to be established (Steinberg *et al.*, 1985).

Regulation of cholesterol metabolism in mammalian cells

Most nucleated mammalian cells have the capacity to synthesize cholesterol from acetyl CoA (Bloch, 1965). The specific factors that regulate flux of cholesterol in specific cells are diverse, but the final mechanisms for regulation of cholesterol synthesis seem to

be similar in most cells (Brown and Goldstein, 1978). In each cell, the regulatory mechanism senses the adequacy of the intracellular pool of cholesterol that is available for metabolic utilization by the cell and adjusts 3-hydroxy-3-methylglutaryl Coenzyme A-reductase (HMG CoA-reductase) activity accordingly (Brown *et al.* 1975). This enzyme is the rate-limiting enzyme in intracellular cholesterol synthesis.

The metabolically active cholesterol pool is governed by a balance of input and output of cholesterol. The input of cholesterol constitutes uptake from circulating lipoproteins and *de novo* synthesis in the cell. The output of cholesterol constitutes utilization for plasma membrane synthesis during cell growth and division and loss of cholesterol from the cell. The cells can lose cholesterol in several ways. These include,

1. loss through passive transfer to plasma lipoproteins from the surface membrane cholesterol
2. active secretion of cholesterol as lipoproteins from cells of the liver and intestinal epithelium and
3. conversion to metabolic products like bile acids in the liver or steroid hormones in endocrine glands.

When the cholesterol output from the cell is constant, there appears to be an inverse relation between the cholesterol input from lipoproteins and intracellular cholesterol synthesis. The differences among different cell types are attributed largely to

1. the nature of the plasma lipoprotein recognized by the cell and
2. the rate of utilization of cholesterol by the cell.

In many cells, when the rate of cholesterol output is increased, both the cellular uptake of cholesterol and cellular synthesis of cholesterol increase. Under these conditions, the LDL receptors will increase and transport LDL cholesterol into the cells (Faust *et al.* 1977).

In addition to free cholesterol, the cells contain esterified cholesterol also. Esterification serves to dampen sudden fluctuations in free cholesterol that occur during sudden alterations in sterol flux. In most cells, the content of esterified cholesterol is much

lower than the content of non-esterified cholesterol. However, in steroid hormone secreting cells, hepatic cells of cholesterol-fed rats and rabbits, and, in phagocytic cells and vascular smooth muscle cells of tissues that have been exposed to an abnormally high influx of plasma proteins such as in xanthomas and atheromas, accumulation of excess of cholesterol esters have been observed (Brown and Goldstein, 1978).

The LDL receptors have been shown to be a major pathway of obtaining cholesterol from the plasma LDL. When LDL receptors are present, cells grown in LDL rich media suppress HMG-CoA reductase activity. In lipoprotein deficient serum, the enzyme activity is increased. This inverse relation between LDL receptors and HMG-CoA reductase activity has been found to be absent in homozygous familial hypercholesterolemia where the LDL receptor pathway is defective (Brown and Goldstein, 1976).

Diet induced hypercholesterolemia in the rabbit

Rabbits clearly become hypercholesterolemic and atherosclerotic also, when placed upon a diet rich in cholesterol. The rabbit absorbs more cholesterol than the rat and retains about 77% of the administered cholesterol. However, the more important reason for the massive increase of cholesterol in the serum in the rabbit was thought to be its peculiar inability to rid its plasma of dietary cholesterol. Thus, in the rabbit, the dietary cholesterol which is absorbed appears to be isolated in the blood. The entry of cholesterol to the liver, where it could be stored, then converted into cholic acid and excreted, is prevented. For these cholesterol carrying chylomicrons to enter the liver they must be made soluble within the plasma by some clearing process or ingested by cells capable of phagocytosis. The hepatic Kupfer cells appear to accomplish this latter function. This Kupfer cell system in the rabbit appears to be deficient in its total mass or in its function (Freidman and Byers, 1954). This is believed to be the main reason for the massive hypercholesterolemia seen in the rabbit after cholesterol feeding. The composition of VLDL lipids from hypercholesterolemic rabbits has been shown to be similar to that of atherosclerotic plaques in rabbits. The uptake of lipoproteins from hypercholesterolemic animals into the aortic

wall is increased compared to VLDL from control animals. This type of evidence has been sited to show that VLDL from hypercholesterolemic rabbits has an inherent atherogenicity (Shore *et al.*, 1974; Rodriguez *et al.*, 1976).

Lipids and atherosclerosis

Chronically elevated concentrations of plasma lipoproteins, particularly LDL and VLDL, have long been associated with increased incidence of atherosclerosis. Since cholesterol in atherosclerotic lesions is derived primarily from cholesterol in circulating lipoproteins, it is reasonable to conclude that hyperlipoproteinemia is causative of atherosclerosis by virtue of its delivery of cholesterol to the artery at a high rate. Thus, high LDL concentrations cause high rates of LDL entry to the artery wall. The increased entry of lipids to the artery wall leads to high rates of uptake by cells of the artery wall or to extracellular trapping. This overloads mechanisms that normally prevent cholesterol accumulation, eventually leading to accelerated atherosclerosis. However, the causative link to atherosclerosis could be different from those discussed above as high plasma LDL is implicated in several other processes involved in atherosclerosis (Steinberg *et al.*, 1985). Some of these are discussed below.

1. Causing damage to endothelial cells. Hypercholesterolemia may induce a subtle form of endothelial "injury". One such form of injury includes alteration in the cholesterol:phospholipid ratio of the plasma membranes. Such changes could lead to increased membrane viscosity which might decrease the malleability of endothelial cells.
2. Influencing migration/metabolism of monocytes and macrophages. Endothelium exposed to hypercholesterolemia may be important in monocyte adhesion and chemotaxis that precedes the development of fatty streaks.
3. Stimulating growth of smooth muscle cells. It is possible that hypercholesterolemia may induce the endothelium to form growth factors which in turn stimulate smooth muscle proliferation.

4. Serving as a source of free fatty acids due to the action of extracellular lipases.

Response to injury hypothesis

In this hypothesis, it is proposed that a form of "injury" to the endothelium precedes the development of atherosclerosis. This injury to the endothelium can be of several forms. The more common form of endothelial injury is loss of isolated cells. This isolated cell loss is rapidly replaced by neighbouring cells (Ross, 1985; Ross, 1986). Replacement may occur by spreading of adjacent cells or if the area is large, by increased turnover of adjacent cells. In the more subtle forms of endothelial injury no morphological manifestations may be detected. However, such endothelial injury may still be sufficient to stimulate endothelial cells to form and secrete growth factors. Endothelial surface alterations could provide opportunities for interaction with platelets and release of growth factors from endothelium, macrophages or platelets. In the presence of endothelial injury, these growth factors have access to the subendothelium. They attract smooth muscle cells from the media into the intima. The proliferation of these smooth muscle cells are believed to lead to the development of atherosclerosis (Ross *et al.*, 1985).

The principal cells involved in atherosclerosis, *viz*, endothelium, smooth muscle, platelets and monocyte/macrophages, either contain or can synthesize and release chemo-attractants and growth factors. One of these is platelet-derived growth factor. It is believed that such growth factors could provide the stimulus for autocrine or paracrine stimulation of smooth muscle proliferation.

According to the response to injury hypothesis, hypercholesterolemia may lead to the development of atherosclerosis by two pathways. In one pathway, monocytes exposed to hypercholesterolemia and platelets interact with the injured endothelium and stimulate plaque formation by release of growth factors. In the other pathway, there is direct stimulation of the endothelium to release growth factors and these can induce smooth muscle migration and proliferation (Ross, 1986). These pathways may be important in

diabetes mellitus, hypertension and cigarette smoking which are associated with increased incidence of atherosclerosis.

VASCULAR SMOOTH MUSCLE CONTRACTION AND RELAXATION

General structure

Smooth muscle cells are fusiform or branched cells approximately 100 - 500 μm long and 2 - 6 μm diameter (Somlyo and Somlyo, 1986). These cells are embedded in a matrix of connective tissue. The connective tissue elements contribute to the distribution of forces generated by the contracting smooth muscle. Coupling between cells occurs through regions of close apposition between the outer leaflets of the plasma membranes of neighbouring cells. A regular lattice of particles called gap junctions are found within these regions of the plasma membrane. These junctions function as low resistance pathways for the rapid spread of electrical signals through the tissue.

Contractile apparatus

Thick filaments (myosin), thin filaments (actin) and intermediate filaments have been identified in vascular smooth muscle. The thick filaments are made up of collections of myosin molecules. Each myosin molecule is made up of two components termed as light and heavy meromyosin. The light meromyosin component forms the tail component and is lined up with other similar molecules to form a thick filament. The heavy meromyosin component appears as protrusions from the thick filaments and these protrusions form cross bridges with actin filaments during contraction. The heavy meromyosin is further fractionated into a globular S-1 segment and a fibrous S-2 segment. The S-1 segment is made up of two globular heads. Each head contains attachment sites for actin, an enzymatic site that hydrolyzes ATP to liberate energy used for contraction and two "myosin light chains" that are involved in the function of these segments. The heavy meromyosin and the light meromyosin appear to form a "hinge" between them. The S-1 segment of the heavy meromyosin transmits the forces generated by the operational site of

the myosin molecule to the light meromyosin (Hartshorne, 1980; Hartshorne and Goreka, 1980).

The thin filaments are made up of contractile proteins called actin and tropomyosin. The regulatory protein called troponin found in skeletal muscle is absent in smooth muscle. The filamentous actin is a two-stranded helix made up of actin monomers and is found in all muscle cells. Actin binds tropomyosin that lies in the groove on either side of the actin filament. The intermediate filaments are not directly involved in the contractile process. These filaments and similar filaments (neuro filaments, glial filaments) are found in several cell types. The energy requirements of smooth muscle are met by aerobic glycolysis and oxidative phosphorylation (Somlyo and Somlyo, 1986).

Excitation-contraction coupling

Electromechanical coupling and pharmacomechanical coupling are two principal forms of excitation-contraction coupling in smooth muscle (Johannason and Somlyo, 1980). These two mechanisms have been shown to operate singly or in combination. The primary trigger for contraction in both mechanisms is a rise in cytoplasmic free Ca^{++}. In electromechanical coupling, contraction is influenced through changes in membrane potential. While depolarization causes contraction by increasing the cytoplasmic free Ca^{++}, hyperpolarization of the surface membrane leads to relaxation. The relative contributions made by intracellular and extracellular calcium ions for contractions vary according to the type of muscle and the stimulus (Godfraind *et. al.*, 1986).

In pharmacomechanical coupling, stimulation of contraction and relaxation is independent of changes in membrane potential. The drug-induced contractions of smooth muscle that are completely depolarized in high potassium solutions and, hence, cannot have their membrane potentials altered by drugs, represent pure pharmacomechanical coupling. The physiologically relevant forms of pharmacomechanical coupling occur together with depolarization when normal, polarized smooth muscles are stimulated by neurotransmitters. Pharmacomechanical coupling has been shown to occur through the release of Ca^{++} from

the sarcoplasmic reticulum. Excitatory transmitters stimulate turnover of phosphatidylinositol in the membrane leading to the generation of inositol triphosphate (1,4,5 isomer). This compound could act as a second messenger releasing Ca^{++} from the sarcoplasmic reticulum (Rasmussen, 1986; Somlyo and Somlyo, 1986).

Regulation of contraction and relaxation

The sliding-filament theory explaining the contraction-relaxation of skeletal muscle is believed to operate in smooth muscles also. The myosin molecules of the thick filaments are arranged in such a manner that the enzymatically active part of the molecule protrudes from the body of the filament. These protruding portions called the cross bridges, bind to actin of the thin filaments during contraction to form sites of tension development. Magnesium-ATP is hydrolyzed during muscle contraction by the enzyme Mg^{++} -adenosine triphosphatase (Mg^{++} ATPase) located in S-1 fraction of heavy meromyosin of the myosin molecule. The maximum shortening velocity appears to be correlated with enzyme activity. It is now generally accepted that activation of smooth muscle actomyosin is initiated by phosphorylation of the light chains of the myosin molecule.

In the relaxed muscle, the sarcoplasmic Ca^{++} is about 100 nmol/l and myosin is non-phosphorylated and there is no cross bridge formation between actin and myosin. An increase in free Ca^{++} in sarcoplasm activates myosin light chain kinase, which phosphorylates the two light chains of the myosin molecule. When the light chains are phosphorylated, actin is able to activate the Mg^{++}ATPase activity of myosin. Hydrolysis of Mg^{++}ATP by this enzyme leads to cross bridge formation and cross bridge cycling between actin and myosin. As long as the sarcoplasmic Ca^{++} is above the activation threshold, cross bridge cycling will continue. When the Ca^{++} level falls, the myosin light chain kinase becomes inactivated. This is associated with myosin dephosphorylation by a phosphatase with reversal of processes (Van Breemen *et al.*, 1980).

The active myosin light chain kinase is composed of two distinct proteins, the smaller of these subunits, calmodulin, has four Ca^{++} binding sites and is the Ca^{++}

receptor of this enzyme. When three of the four Ca^{++} binding sites of calmodulin are occupied, the Ca-calmodulin complex interacts with the larger subunit of the enzyme to form active myosin light chain kinase. Myosin light chain kinase can itself be phosphorylated at two sites by cyclic-AMP dependent protein kinase. The activity of myosin light chain kinase declines when both sites are phosphorylated, and it has been suggested that phosphorylation of myosin light chain kinase by the cyclic-AMP dependent protein kinase may play a role in β adrenergic relaxation of smooth muscle. Myosin light chain phosphatases dephosphorylate the phosphorylated myosin. These enzymes appear to be active during both contracted and relaxed states of smooth muscle. When the sarcoplasmic Ca^{++} is high, myosin light chain kinase activity overrides the activity of the phosphatases. When the Ca^{++} levels are low, the kinases are inhibited and the activity is shifted in favour of the phosphatases.

Polyphosphoinositides and vascular smooth muscle contraction

Our understanding of the regulation of Ca^{++} in smooth muscle has increased significantly following the discovery of polyphosphoinositides. These compounds have been shown to function as second messengers (Rasmussen, 1986). The occupation of certain cell surface receptors (e.g., α_1-adrenoceptors in vascular smooth muscle) by agonists leads to the activation of a specific enzyme called phospholipase C. The activated enzyme phospholipase C, causes hydrolysis of membrane phospholipids leading to the generation of inositol triphosphate (IP_3) and diacylglycerol (DG). The release of inositol triphosphate (1, 4, 5 isomer) leads to mobilization of Ca^{++} from the endoplasmic reticulum and rise in cytosolic free Ca^{++}. This Ca^{++} combines with calmodulin and the Ca^{++}-calmodulin complex activates the enzyme myosin light chain kinase. Activation of this enzyme has been known to lead to vascular smooth muscle contraction.

The contractile response elicited by an agonist can be divided into two components. Of these two components of the contraction, the initial phasic component is thought to be due to the intracytoplasmic release of Ca^{++}. The slow sustained tonic phase of the

contraction is thought to be due to the continued entry of Ca^{++} into the cell from outside. It is now believed that the initial phasic component of the contraction is mediated by inositol triphosphate and the slower tonic component by diacylglycerol (Abdel-Latif, 1986). With continued occupation of receptors, both limbs of the inositol pathway (inositol triphosphate and diacylglycerol) are activated. With continued formation of diacylglycerol, there is continued entry of Ca^{++} into the cell and the contractile response is maintained. These processes can be terminated by removal of the receptor-agonist interaction ultimately leading to relaxation of vascular smooth muscle.

Cyclic nucleotides and vascular smooth muscle relaxation

The cyclic nucleotides, cAMP and cGMP have been known to cause vascular smooth muscle relaxation. Although changes in cAMP are seen in many instances of contraction and relaxation, convincing evidence that cAMP is causing the relaxation has been shown only for a few agents. These agents include isoprenaline, adenosine and prostacyclin (Kukovetz *et al.*, 1981). The following criteria are used to establish that the relaxation produced by a specific agent as being mediated by cAMP.

1. The agent causes a rise in cAMP.
2. Close parallelism between cAMP levels and relaxation.
3. Effector response potentiated by phosphodiesterase inhibitors.
4. Actions mimicked by dibutyryl cAMP.

During β adrenoceptor mediated vascular smooth muscle relaxation by isoprenaline, the cAMP is believed to mediate its effects by activation of cAMP dependent protein kinases. This function had been described previously. However, in addition, cAMP could decrease sarcoplasmic Ca^{++} by increasing Ca^{++} uptake into the sarcoplasm reticulum and increase of Ca^{++}extrusion through the sarcolemma (Mueller and Van Breemen, 1979). The decreased membrane excitability due to hyperpolarization of the cell membrane helps in the relaxation process also.

Some vasoactive agents produce vasodilatation through changes in cGMP concentrations. Such agents include nitrovasodilators and EDRF (Kukovetz *et al.*, 1983; Rapoport and Murad, 1983). The relaxations mediated by these agents have been attributed to cGMP for the following reasons.

1. Time course experiments using several nitro compounds have shown that changes in cGMP always preceded relaxation (Kukovetz *et al.*, 1979).
2. The effects of cGMP mimicked by 8-bromo-cGMP.
3. The effector responses were potentiated by the cGMP phosphodiesterase inhibitor, M & B 22,948.

Although it is likely that cGMP lowers intracellular free Ca^{++}, the exact mechanism is uncertain. Similar to cAMP, the entry of Ca^{++} into intracellular organelles (mitochondria and microsomes) and Ca^{++} extrusion through the cell membrane may be increased.

METHODS

GENERAL METHODS

Animals and Diets

Male, New Zealand White rabbits purchased from a local vendor were used in the study. At the time of weaning (age 8 weeks, 1.5 - 2.5 kg body weight), the animals were brought to the vivarium, numbered and housed individually. Except where special diets were involved, the rabbits were given a standard rabbit diet (Baby Rabbit Pellets, M-0662, Maple Leaf Mills Ltd., Masterfeeds Division, London, Ontario, Canada) and were weighed at regular intervals.

For induction of atherosclerosis, the rabbits were fed a high lipid, high cholesterol (cholesterol 2% w/w) diet (5799C-9, Rabbit Purified Diet, Ralston Purina Co., Richmond, Indiana, USA). This diet will be referred to as the 2% cholesterol diet throughout the text. The compositions of the two types of diets used in the study are given in Table 1. The ingredients used in the formulation of the diets are given in Appendix 1. Animals randomized to the 2% cholesterol diet will be referred to as the experimental animals. A separate group of age matched rabbits was always selected at this time to go on the standard rabbit diet. These animals will be referred to as the control animals. Food and water were given *ad libitum* to both groups of animals. The food intake was monitored by weighing the residual food at regular intervals. More animals than the required numbers were assigned to the two groups to compensate for any deaths during the study. Blood was taken from the central ear artery of the animals at regular intervals for estimation of serum cholesterol and triglycerides.

Anesthesia and dissection of the rabbit

The animals were anesthetized by injecting a solution of sodium pentobarbitone (25 mg/kg) through the marginal ear vein. A midline thoracotomy was performed. A sample of blood was taken from the heart, centrifuged at 2000 rpm for 10 minutes and the serum stored at -70°C pending analysis. The thoracic viscera were pushed to the right side of the

Table 1. The compositions of the control and the experimental diets are given below. The percentages of the individual fatty acids in the lipids of these two diets are given in the lower part of the table.

	Control diet (w/w)	Experimental diet (w/w)
Proteins	17.9%	18.4%
Lipids	4.0% (cholesterol 0.06%)	24.0% (cholesterol 2.0%)
Carbohydrates, minerals and vitamins	78.1%	57.6%
Fatty acid		
16:0 Palmitic acid	17.5	25.0
18:0 Stearic acid	2.5	12.6
18:1 Oleic acid	16.8	44.4
18:2 Linoleic acid	43.7	10.3
18:3 Linolenic acid	16.4	0.5
Others	3.1	7.2

chest, and the inferior vena cava cut between clamps. The thoracic aorta was identified and cut between clamps at the point it pierced the diaphragm. The proximal clamp holding the cut end of the aorta was lifted up and the aorta was separated from the prevertebral tissues. The carotid arteries and the trachea were cut transversely and the whole mediastinum was lifted off by cutting the other attachments. Care was taken to avoid unnecessary stretching or trauma to the aorta. The excised aorta with the other mediastinal organs was immediately placed in a large beaker containing oxygenated, cold, Krebs-bicarbonate buffer of the following composition (mmol/l); NaCl 116.0, KCl 5.4, $CaCl_2$ 1.2, $NaHCO_3$ 22.0, NaH_2PO_4 1.2, $MgCl_2.6H_20$ 1.2, glucose 10.1, $CaNa_2EDTA$ 0.023. The abdominal aorta was dissected and preserved for estimation of cholesterol in aortic tissue.

Preparation of aortic rings

The mediastinal organs were next transferred to a dissecting tray containing the Krebs-bicarbonate buffer. The aorta was dissected free and cut transversely at its root. The remaining viscera were discarded and the dissecting tray replaced with fresh buffer. The aorta was pinned at the two ends, inspected and the excess adventitial fat and connective tissue removed carefully. The long column of clotted blood inside the aorta was gently pulled out with forceps. This usually came out as a single column. Aortic rings (approximately 5 mm long) were prepared by cutting transversely across the aorta with a pair of sharp scissors. The first ring was cut below the origin of the left subclavian artery. When the rings were cut, an attempt was made to keep them of equal size (Tallarida and Jacob, 1979).

Tissue bath technique

Each aortic ring was mounted between two triangular metal clips and suspended in a tissue bath of 22 ml capacity containing Krebs-bicarbonate buffer. A diagrammatic representation of the tissue bath used is shown in Figure 2 (Senaratne, 1984). The tissue bath was kept at 37°C with the aid of a heater circulator (Model No. E 15, Haake Mess-Technik Gmbt U.Co, Karlsruhe, Federal Republic of Germany) which circulated heated

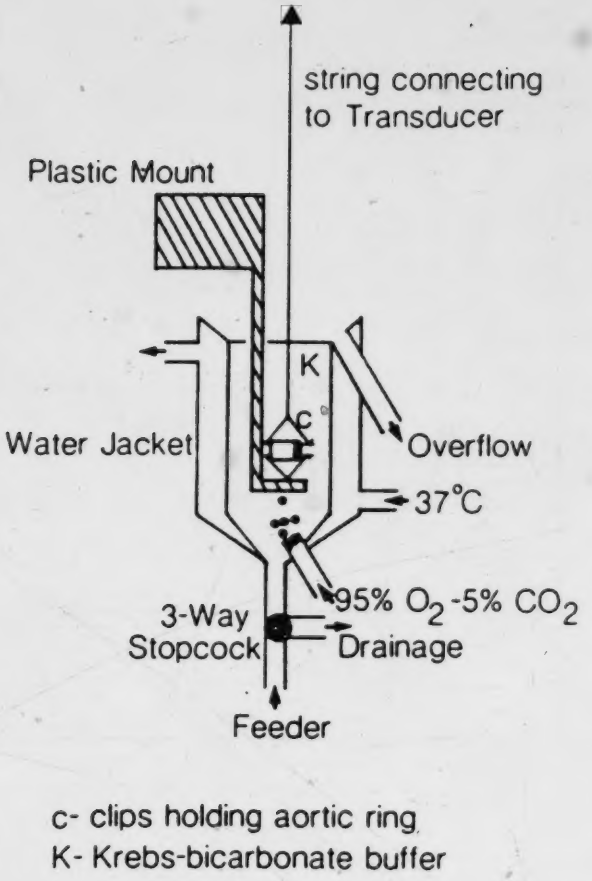

Figure 2: A diagrammatic representation of the tissue bath used for experiments on the isolated rings of rabbit aorta in the present study.

water through the water jacket in each tissue bath. The Krebs-bicarbonate buffer (pH 7.3 - 7.4) in the tissue bath was maintained at 37°C and continuously aerated with a gas mixture containing 95% O_2 and 5% CO_2. Fresh buffer was fed into the tissue bath from a reservoir which was also aerated and maintained at 37°C. The bath fluid was changed with the aid of feeder and drainage tubes and the bath volume was kept constant by an overflow tube. Both the drainage and overflow tubes were connected to a vacuum apparatus to ensure complete drainage. An assembly of eight tissue baths was used in the experiments described in this thesis. Of the triangular metal clips used to suspend the aortic ring, the lower clip was attached to a moveable plastic mount. This enabled fine adjustments to be made in the lengths of the aortic rings. The upper clip was connected to a force displacement transducer (Model No. FT .03C, Grass Instrument Co., Quincy, Mass., USA) for isometric tension recording. The outputs of the transducers were amplified and recorded (Recorder Model No. 2400S, Gould Inc., Cleveland, Ohio, USA). A fine silk thread (No. 5-0) was used for all attachments.

Determination of optimum basal tension

At the beginning of the study, a length-active tension curve was performed to determine the optimum basal tension for the rabbit aorta (Wyse, 1980). This involved the determination of the basal tension at which the contractile response to a fixed concentration of noradrenaline (-7.0 log mol/l) was a maximum. Starting from a length at which the tension was zero, the tension of the preparation was increased by 1 g every 5 minutes. Following 2 minutes of stabilization at each tension, the preparation was exposed to a fixed concentration of noradrenaline. With an increase in initial basal tension the response to noradrenaline increased. This relationship continued until the response to noradrenaline reached a plateau. Beyond a certain basal tension, the response to noradrenaline decreased if the tension was increased further. The optimum tension was defined as the basal tension at which the magnitude of the response to noradrenaline reached a maximum.

These experiments showed that the optimum basal tension for the rabbit aorta was about 8.0 grams (g).

Hence, after mounting the aortic rings, they were stretched to a basal tension of 8.0 g, gradually over the first 30 minutes. The preparations were equilibrated for a total of 90 minutes before the experiment proper was begun. During this period, the tissue bath fluid was replaced with fresh buffer every 30 minutes.

Concentration-effect curves

Concentration-effect curves to the adrenoceptor agonists were obtained in a cumulative manner, graded doses being added to give the desired concentration of the drug in the tissue bath (Van Rossum *et al.*, 1963). The total volume of solution added to the tissue bath during a cumulative concentration-effect curve was less than 4% of the total bath volume. Concentrations of the agonists were increased in half log units each time. Each dose was added when the responses to the preceding dose had reached a plateau. After obtaining a complete concentration-effect curve, at least 45 minutes was given for the tissue to recover before a second concentration-effect curve was started.

Removal of endothelium

In some aortic rings, the endothelium was deliberately removed by mechanical means. For this purpose the tip of a small tissue forceps was inserted to the luminal surface of the aortic ring and turned back and forth for 20 seconds on a filter paper wetted with Krebs-bicarbonate buffer (De Mey and Vanhoutte, 1982). Such rings are referred to as de-endothelialized rings in the text. Scanning electron microscopy of the luminal surface of these rings showed that this procedure produced complete or near complete denudation of aortic endothelium.

Estimation of Cholesterol and Triglycerides

The concentrations of cholesterol and triglycerides in the serum were estimated using an automated system (Multistat III, Instrumentation Laboratories, Lexington, KY, USA) that incorporated the methods of Allain *et al.* (1974) and Pinter *et al.* (1967)

respectively. The tissue cholesterol estimations were performed in the abdominal aorta by the method described by Morin (1976). The tissue protein estimations were made using the method described by Hartree (1972).

Sudan Red staining

Individual aortic rings from the tissue bath experiments and long segments of donor aortae obtained after bioassay experiments (*vide infra* protocol 2.2) were subjected to sudan red staining. The specimens were slit open and pinned on pieces of cork exposing the intimal surface. These were rinsed in 70% ethyl alcohol for 1-2 minutes and immersed in sudan red solution at room temperature for 15 minutes. The intimal surface was kept submerged in the stain. The solution was agitated intermittently at this stage. Next, the specimens were transferred to 80% ethyl alcohol for 20 minutes and washed in running water for 1 hour. Finally, the tissues were stored in 10% buffered formalin (Holman *et al*. 1958). The methods of preparation of solutions used in this section are given in Appendix 2.

Grading of Sudanophilia

The extent of experimental cholesterol atherosclerosis (in the descending thoracic aorta) shown by the sudanophilia in each animal was visually graded on a scale of 0 to 4. The method of grading adapted was a modification of the methods described by Duff and McMillan (1949) and by Kritchevsky *et al*. (1978).

Grade 0 - no lesions seen in the aorta.

Grade 1 - lesions around orifices of intercostal arteries, surface involvement less than 5%.

Grade 2 - lesions between orifices of intercostal arteries in addition to Grade 1 lesions, total surface involvement between 5-25%.

Grade 3 - confluent lesions present, total surface involvement 25-60%.

Grade 4 - confluent lesions present, total surface involvement over 60%.

Light Microscopy

The specimens used were pieces of aorta from control and experimental animals obtained before and after tissue bath and bioassay experiments. The tissues were fixed in 10% buffered formalin for 24 hours. For lipid staining, the specimens were washed in Millonig's phosphate buffer for 1 hour and postfixed in 1% osmium tetroxide in Millonig's buffer for 2 hours. The specimens were then transferred through a series of graded ethyl alcohol solutions (70, 80, 95 and 100% for 1 hour in each stage) and embedded in paraffin (Tissue Prep, 56°C). For hematoxylin-eosin staining, the tissues were transferred from 10% formalin to the alcohol dehydration state (as above) and embedded in paraffin. Sections were cut at 10 µm on a rotary microtome (Model No. 820, American Optical Co., Belville, Ontario, Canada) and stained by the methods modified from those described by Humason (1979) and Culling (1976) and examined under a light microscope. All chemicals and reagents used in this section were obtained from Fisher Scientific Company (Fair Lawn, NJ, USA).

Scanning Electron Microscopy

Scanning electron microscopy was done on two groups of specimens.

1. Specimens taken from control and experimental animals after fixation by pressure perfusion. These animals were used only for anatomical studies.
2. Specimens taken before and after tissue bath and bioassay experiments without fixation by pressure perfusion.

Pressure perfusion method (Russel and Amy, 1986)

After anesthesia, the chest was opened and the aorta was perfused through a cannula inserted into the left ventricle. The tissues were perfused with 1 L of Tyrode's solution kept at a pressure of 100 mmHg. Later, the perfusion medium was changed to Tyrode's solution containing 2.5% glutaraldehyde (electron microscopy grade) and the perfusion continued with a further 1 L. This procedure allowed the entire animal to be fixed with the shape of the arteries intact. The arteries were dissected and placed in 2.5%

glutaraldehyde in Tyrode's solution for 5 days. The pieces of arteries were processed for scanning electron microscopy as described below.

The rabbit aortic rings were fixed initially with 2.5% glutaraldehyde in Millonig's buffer (48 hours) followed by post fixation with 1% osmium tetroxide (45 minutes). The specimens were dehydrated using ethyl alcohol (15 minutes in 50, 70, 80 and 95% alcohol followed by three 10 minute periods in 100% alcohol) and then placed in a critical point dryer (Model No. LPD 100, see VAC, Pittsburg, Penn, USA) for 5 minutes at 41°C and 1200 psi CO_2. The tissues were then mounted in aluminum stubs with silver glue, sputter-coated with gold, and examined in a scanning electron microscope (Model No. 505, Philips, Eindhoven, The Netherlands). These methods are modifications of those described by Glauert (1975) and Dawes (1981).

Transmission Electron Microscopy

As discussed under scanning electron microscopy specimens from animals fixed by pressure perfusion as well as after regular tissue bath and bioassay experiments were used in these studies. The tissues were fixed initially with 2.5% glutaraldehyde in Millonig's buffer (24-48 hours). They were then washed three times (15 minutes each) with Millonig's buffer and post-fixed for 1 hour in 1% osmium tetroxide in Millonig's buffer. After washing for three 10 minute periods with double distilled water, the tissues were dehydrated through a graded series of ethyl alcohol solutions (as described under scanning electron microscopy). Samples were transferred from absolute alcohol to propylene oxide for three 10 minute periods and then into a 1:1 mixture of propylene oxide and araldite (CY 212, Marivac Limited, Halifax, N.S., Canada) epoxy resin for 3-4 hours. They were then placed in pure resin in embedding blocks, left at room temperature overnight, and then at 60°C in an oven for 48 hours (Hayat, 1981). Thin sections were cut on an ultramicrotome (Reichert Jung Ultracut E, C. Reichert Ag, Vienna, Austria), and mounted on 300 mesh copper grids. The specimens were then counterstained with uranyl acetate and lead citrate and examined in a transmission electron microscope (Model 410,

Philips, Eindhoven, The Netherlands). The methods of preparation of solutions used in the electron microscopy studies are given in Appendix 2.

SPECIFIC METHODS

The specific protocols carried out are listed below.

PROTOCOL ONE-CHOLESTEROL FEEDING AND EDR

Protocol 1.1. Determination of the effect of 2% cholesterol diet given for 4, 8 and 10 weeks on EDR to acetylcholine. The lesions produced in the aortae at the different stages of feeding were studied also.

Protocol 1.2. Determination of the effect of subsequent reversal to standard rabbit diet for 14 and 32 weeks, after an initial 6 week period of 2% cholesterol diet, on EDR to acetylcholine.

Protocol 1.3. Determination of the effect of 2% cholesterol diet on the responses of rabbit aorta to α adrenoceptor agonists.

PROTOCOL TWO-BIOASSAY FOR EDRF

Protocol 2.1. Development of a bioassay for EDRF.

Protocol 2.2. Determination of synthesis/release of EDRF in aortae from control and cholesterol-fed rabbits.

Protocol 2.3. Determination of synthesis/release of EDRF in rabbits reversed to standard rabbit diet for 36 weeks after an initial 6 weeks of cholesterol feeding.

PROTOCOL THREE-EFFECT OF AGE AND CHOLESTEROL FEEDING ON EDR

Protocol 3.1. Determination of the combined effect of age and 2% cholesterol diet on EDR to acetylcholine.

PROTOCOL FOUR-CALCIUM CHANNEL BLOCKERS AND EDR

Protocol 4.1. Determination of the effect of chronic oral administration of the calcium channel blocker, nicardipine, on EDR to acetylcholine.

Protocol 4.2. Determination of the effect of incubation of rabbit aorta *in vitro* with nicardipine and diltiazem on EDR to acetylcholine.

Protocol 4.3. Determination of the effect of nicardipine on synthesis/release of EDRF and on the effects of EDRF in rabbit aorta.

Protocol 4.4. Determination of the effects of nicardipine and diltiazem on contractions induced by potassium.

PROTOCOL FIVE-DIABETES AND EDR

Protocol 5.1. Determination of the effect of diabetes mellitus on EDR to acetylcholine.

PROTOCOL ONE-CHOLESTEROL FEEDING AND EDR

The design of the study is shown in Figure 3. In this protocol, the EDR to acetylcholine was assessed in cholesterol-fed animals at 0, 4, 8 and 10 week intervals. After completing the study, the aortic tissues were stained with sudan. Their histologic (light microscopy, scanning and transmission electron microscopy) appearances were studied at these different stages also. Some animals were processed for scanning electron microscopy after pressure perfusion. The EDR to acetylcholine was assessed in a group of animals where the diet was reversed to standard rabbit diet after an initial 6 weeks of cholesterol feeding also. In addition, the responses to α adrenoceptor agonists at different stages of cholesterol feeding were investigated.

Protocol 1.1

In this protocol, the EDR to acetylcholine at 0, 4, 8 and 10 week intervals after cholesterol feeding were studied. A group of 6 animals were killed at the beginning of the study (0 weeks). Subsequently, 6 animals from control and experimental groups were killed at 4, 8 and 10 week stages of the study.

Experimental Protocol

The responses to acetylcholine were examined in 4 aortic rings from each animal with each ring being assigned randomly to one of the following procedures.

1. Control with no additional manipulation
2. Incubation with indomethacin (-6.0 log mol/l)
3. Incubation with atropine (-8.0 log mol/l)

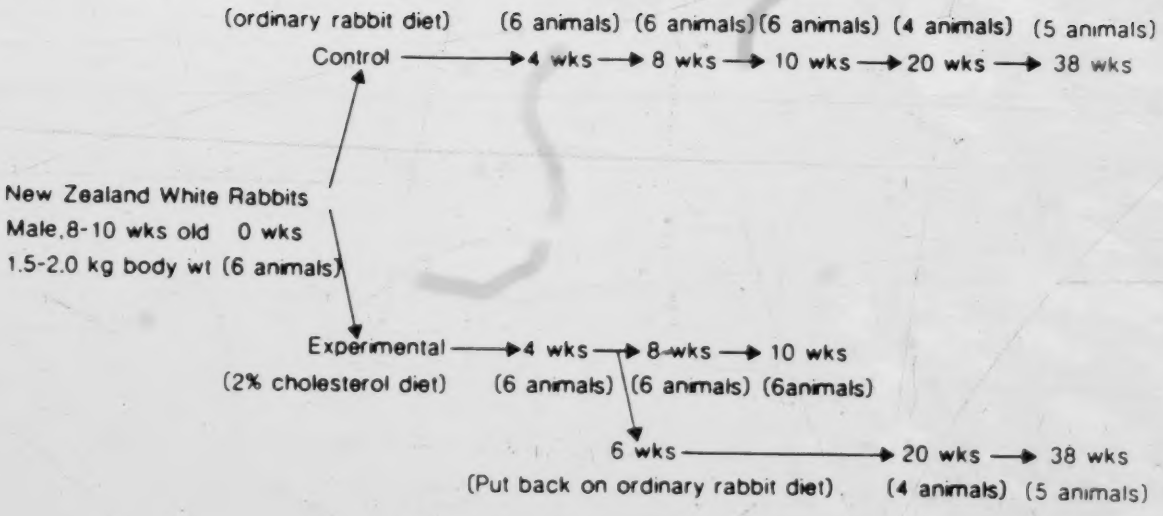

Figure 3. The design of Protocol One is shown schematically. The number of animals shown in parentheses were killed at each stage.

4. Removal of endothelium.

As described under GENERAL METHODS, the aortic rings were equilibrated for 90 minutes before the experiments were begun. At the end of 60 minutes of equilibration, indomethacin and atropine were added to the respective tissue baths. Each drug was left in its tissue bath for 30 minutes and allowed to equilibrate. At the end of this 30 minute period (total of 90 minutes of equilibration), all preparations were contracted by adding noradrenaline (-6.0 log mol/l). After the contractions reached a plateau, concentration-effect curves to acetylcholine were obtained by adding the drug to the bath in a cumulative manner (-9.0 to -4.0 log mol/l). Indomethacin and atropine were present in the respective tissue baths throughout the experiments. On completion of the concentration-effect curves, the tissue bath fluid was replaced with fresh Krebs-bicarbonate buffer and the tension allowed to return to the baseline value with frequent replacement of the buffer. After a period of 45 minutes, the concentration-effect curves to acetylcholine were repeated in the control preparation with the tissue contracted by adding a lower concentration of noradrenaline (-7.0 log mol/l). This enabled the EDR to be assessed when the initial active tension was lower than that elicited by -6.0 log mol/l of noradrenaline.

Effects of NDGA, Quinacrine and Hydroquinone

The effect of nordihydroguaiaretic acid (NDGA) on the response to acetylcholine was assessed by adding it to the tissue bath as a single dose (-4.4 log mol/l) when the relaxation response to acetylcholine was maximal (concentration range -6.0 to -5.5 log mol/l). In separate rings, the effects of quinacrine (-6.0 log mol/l) and hydroquinone (-4.0 log mol/l) on the EDR to acetylcholine were assessed also. These agents were also added to separate tissue baths as single doses when the relaxation responses to acetylcholine were maximal. Effects of quinacrine and hydroquinone were assessed only in the first group of 6 animals killed at the beginning of the study.

Effect of sodium nitrite

The effect of sodium nitrite was investigated in rings with and without endothelium isolated from rabbits at the 10 week stage of the study. As in the initial part of the protocol, the rings were precontracted with noradrenaline (-6.0 log mol/l) and sodium nitrite was added to the tissue bath in concentrations of -4.0 and - 3.0 log mol/l. At the end of each experiment, some rings were prepared for histological studies. The remaining rings were stained with sudan red.

Protocol 1.2 (reversal study).

This protocol was designed to determine the effect of reversing to standard rabbit diet after an initial period (6 weeks) of cholesterol feeding on EDR to acetylcholine. After being on the 2% cholesterol diet for 6 weeks, some animals were randomly selected and transferred back to standard laboratory rabbit diet. These animals, along with their age matched controls, were killed after 14 and 32 weeks of reversing of the diets, and EDR to acetylcholine was assessed in tissue baths as described in protocol 1.1. Such animals, where the diet was changed to standard rabbit diet after an initial period of 2% cholesterol feeding, are referred to as reversal animals in the text.

Protocol 1.3.

This protocol was designed to determine the effect of 2% cholesterol feeding on responses to α-adrenoceptor agonists. The non-selective α-adrenoceptor agonists noradrenaline and clonidine and the selective α_1 adrenoceptor agonist methoxamine were used in the study. The responses to these drugs were assessed as concentration-effect curves. In the three tissue baths where these three adrenoceptor agonists were tested, the β-adrenoceptor antagonist propranolol (-6.0 log mol/l) was incorporated in the Krebs-bicarbonate buffer 30 minutes before the adrenoceptor agonists were added. Concentration-effect curves to the agonists were obtained in aortic rings from control and experimental animals at 0, 4, 8 and 10 week stages of the study. This study was extended to the aortic rings obtained from reversal animals also.

PROTOCOL TWO-BIOASSAY FOR EDRF

In Protocol One, the EDR to acetylcholine was studied in tissue baths. Acetylcholine stimulates receptors on the endothelium to synthesize/release EDRF and this in turn diffuses to the underlying smooth muscle mediating relaxation (Furchgott, 1983). In the tissue bath the two stages of synthesis/release of EDRF and the relaxation effects of EDRF could not be separated. Hence, a bioassay technique was developed which helped to investigate synthesis/release of EDRF and the effects of EDRF separately.

Protocol 2.1.

Development of the bioassay for EDRF is described here. A modification of the techniques described by Griffith *et al.* (1984) and Rubanyi *et al.*, (1985) were used in the study. The assay was based on transferring the factor (EDRF) released from a donor aortic tissue onto a deendothelialized ring of recipient aorta, in which its biological activity could be monitored. A rabbit was anesthetized and a 4-5 cm length of descending thoracic aorta removed as the donor tissue. A separate 5 mm long ring of aorta was used as the recipient. This recipient was de-endothelialized as described previously. A diagrammatic representation of the bioassay apparatus is shown in Figure 4A. The donor aorta (d) was placed in a rectangular plastic chamber (C, 10 cm × 10 cm × 3 cm) which was enclosed in a water jacket and filled with Krebs-bicarbonate buffer. The opposite walls of this chamber were pierced by two stainless steel cannulae (outer diameter 3.2 mm, inner diameter 2.2 mm) whose positions could be adjusted according to the length of the donor aorta. The donor aorta was mounted inside the chamber between the ends of the two cannulae. This donor aorta was perfused intra-luminally with oxygenated Krebs-bicarbonate buffer (95% O_2-5% CO_2; pH 7.4) from a reservoir (R) by means of a positive displacement roller pump (P, Miniplus 2, Gilson Medical Electronics, Villiers, France). A multiple channel infusion pump (Ac, Model No. 2212, Harvard Instrument Co. Inc., Newport Beach, CA, USA) was used to introduce Krebs-bicarbonate buffer or acetylcholine into the perfusate at a point proximal to the donor. The perfusate emerging from the chamber (C) was dripped over the recipient ring of rabbit aorta (r) which was connected to a force displacement

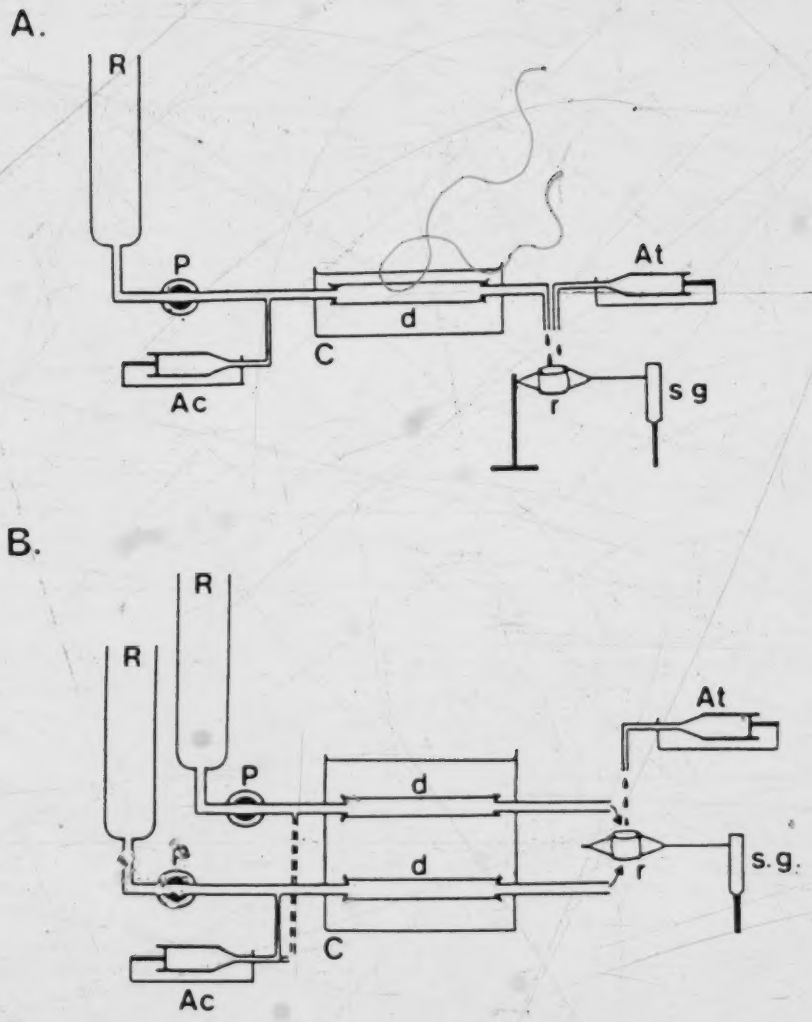

Figure 4.
A: Diagrammatic representation of the apparatus used for bioassay experiments. R, reservoir for Krebs-bicarbonate buffer (maintained at 37°C); d, donor tissue; r, recipient tissue (de-endothelialized); sg, strain gauge for isometric tension recording; Ac, syringe pump for injecting acetylcholine into perfusate; At, syringe pump for injecting atropine directly onto the recipient. The donor is perfused intraluminally and the perfusate is directed onto the recipient (r).
B: The modified apparatus where responses of two donor tissues (d) from control and atherosclerotic animals could be compared.

transducer for recording isometric tension. A second infusion pump (At, Model No. 301A, Sage Instruments, Cambridge, Ma, USA) was used to infuse atropine to the superfusate as shown in Figure 4A. Thus, the recipient tissue alone was treated with atropine. With the aid of the three-way stopcock placed between the reservoir (R) and the tissue chamber, it was possible to introduce a second reservoir as an alternate source of perfusate. The reservoirs and the tissue chamber were maintained at 37°C with the aid of a heater/circulator (Model No. E 15, Haake Mess Technik Gmbt.U.Co., Karlsruhe, Federal Republic of Germany). During the experiments, the chamber C was drained through an outlet at the side and filled every 45 minutes with fresh Krebs-bicarbonate buffer kept at 37°C.

The flow rates of the pumps were set as follows. The positive displacement roller pump P was set to deliver 2.3 ml/min of Krebs-bicarbonate buffer from the reservoir. The multiple channel infusion pump Ac delivered 0.45 ml/min of either Krebs-bicarbonate buffer or increasing concentrations of acetylcholine into the perfusate. The second pump At delivered atropine at a rate of 0.25 ml/min. Thus, the total flow of superfusate onto the recipient was maintained constant at 3.0 ml/min. All the connections in the apparatus were made with polyethylene tubing.

A modified form of the bioassay apparatus in which the responses elicited in a common recipient by two donors could be compared as shown in Figure 4B. In this modification, the chamber C was partitioned into two sections. A donor aorta from a control animal was mounted in one section while the donor aorta from a cholesterol-fed animal was mounted on the other section. Hence, the perfusate through either control or atherosclerotic donors could be dripped on the recipient tissue. Alternatively, the recipient could be obtained from either control or atherosclerotic animals.

Experimental Protocol

The donor aorta was mounted and perfused with Krebs-bicarbonate buffer and a period of one hour was allowed for equilibration. After mounting the recipient tissue, the basal tension was set by stretching the vessel over the first 30 minutes. During the

equilibration period, the recipient was superfused with buffer going through the donor. After one hour, the perfusate was changed to Krebs-bicarbonate buffer containing noradrenaline (0.20 µmol/l) delivered from a second reservoir and atropine (10 µmol/l) was added to the recipient from the pump At. The noradrenaline in the perfusate produced a contractile response in the recipient. After the contraction in the recipient had reached a plateau the perfusate from the infusion pump Ac was changed from Krebs-bicarbonate buffer to progressively increasing concentrations of acetylcholine. The applications of acetylcholine were interspersed with infusions of Krebs-bicarbonate buffer from the same pump.

Modifications for validation of the technique

The following modifications to the basic protocol were completed in order to validate the endothelium-dependent nature of the relaxation of the recipient ring.

1. Substitution of carbachol (5.0 µmol/l) or calcium ionophore A 23187 (1.5 µmol/l) in place of acetylcholine.
2. Exposure of the donor tissue to atropine (0.8 µmol/l) for 30 minutes before challenge with acetylcholine.
3. Removal of endothelium from the donor. This was achieved by inserting a cotton swab on a wire, into the lumen of the aorta and rolling it inside several times.
4. Application of acetylcholine directly onto the recipient without contact with the donor.

Calculations

The concentration of noradrenaline (0.20 µmol/l) in the reservoir R was diluted 1.304 times by the fluid entering from the infusion pumps (Ac and At) resulting in a final noradrenaline concentration of 0.15 µmol/l at the recipient. The acetylcholine infused (0.45 ml/min) into the perfusate (2.3 ml/min) was diluted approximately six times. The concentrations of acetylcholine in the infusion pumps were 3.2, 10.0, 31.6 and 100.0 µmol/l, and after dilution the respective concentrations achieved in the fluid perfusing the

donor were 0.5, 1.6, 5.3 and 16.6 µmol/l . Atropine infused onto the recipient was diluted 12 times. The concentration of atropine in the pump At was 10 µmol/l and after dilution, the final concentration on the recipient was 0.83 µmol/l.

Protocol 2.2

This protocol compared the synthesis/release of EDRF from aortae obtained from control and cholesterol-fed animals. The design of the protocol is shown in Figure 5. The modified apparatus shown in Figure 4B was used for this protocol. The responses elicited in a common recipient by two donors were compared. In addition, this arrangement permitted the recipients to be changed as required.

New Zealand White rabbits (body weight 2.0 - 2.4 kg, age 8 - 10 weeks) were assigned randomly to control and experimental groups. The control group was given the standard rabbit diet and the experimental group was given the 2% cholesterol diet. Animals from each group were killed after being on the respective diets for 6 weeks and the aortae were prepared for bioassay of EDRF.

Three series of experiments were done in this protocol. In the first series, the donor aortae were obtained from control and cholesterol-fed animals. The recipient tissues were obtained from control animals. In the second series, the donor aortae were obtained from control and cholesterol-fed animals and the recipient tissues were obtained from cholesterol fed animals. In the third series a single donor tissue and two recipient tissues were used. The donor tissue was obtained from control animals. One recipient tissue was obtained from the control animals and the other from cholesterol-fed animals. Initially, the response in the recipient tissue from the control animal was obtained. Next this tissue was removed and the recipient obtained from the cholesterol-fed animal was mounted and its response against the same donor tissue was obtained.

In this part of the study the following sequences of concentration-effect curves relating EDR to acetylcholine were obtained.

1. Control response with acetylcholine perfusing the control donor

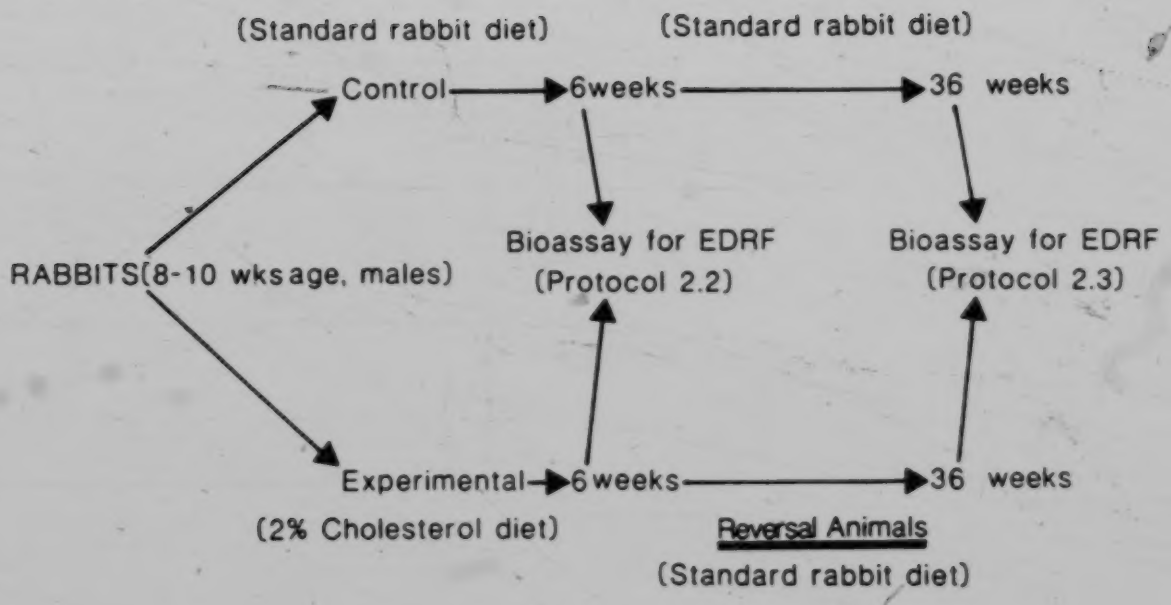

Figure 5. The design of Protocols 2.2 and 2.3 are shown schematically.

2. Test response with acetylcholine perfusing the atherosclerotic donor

3. Control response repeated with acetylcholine perfusing the control donor.

As no significant differences were seen in the relaxations when acetylcholine was perfused through the control donor (responses 1 and 3), the mean relaxations of these two curves were compared with the test response.

In the third series of experiments mentioned above, the responses elicited in two recipient tissues (against a single control donor) were compared. In this experiment, acetylcholine was perfused through the control donor and the response in the recipient tissue obtained from the control animal was determined first. Next this tissue was removed and the recipient from the cholesterol fed rabbit was mounted. After 60 minutes of equilibration, acetylcholine was perfused through the control donor and the response in the recipient tissue was determined as before.

After completion of this part of the study, the remaining animals from the experimental group were transferred to the same diet as that fed to the control animals. These animals were followed up for a further 36 weeks. At the end of this period, these animals along with their age matched controls were killed and aortae prepared for the bioassay (Protocol 2.3).

Protocol 2.3.

This protocol compared synthesis/release of EDRF in aortae obtained from control and experimental animals. The experimental animals were on standard rabbit diet for 36 weeks after an initial 6 week period of cholesterol feeding. The control animals were fed the standard rabbit diet throughout. The design of this protocol is shown in Figure 5. The modified apparatus with two channels shown in Figure 4B was used for the bioassay. Aortae from the two groups of animals were mounted as described previously. The recipient ring was taken from a separate group of New Zealand White rabbits (10-12 weeks of age; weight 2.3 ± 0.2 kg; mean ± s.e. mean). Preliminary studies with recipient rings of aorta taken from animals in the control and experimental groups (i.e., older animals)

failed to produce consistent contractions with noradrenaline and were deemed unsuitable for use as recipients in the bioassay. The responses to acetylcholine were assessed as described previously (Protocol 2.2)

PROTOCOL THREE-EFFECT OF AGE AND CHOLESTEROL FEEDING ON EDR.

It has been reported that older rabbits have a lesser susceptibility to develop hypercholesterolemia and atherosclerosis when fed on diets supplemented with cholesterol (Harman, 1962). However the influence of age on the impairment of the EDR produced by a high cholesterol diet has not been investigated. Therefore, this protocol was designed to test the hypothesis that the age of the animal influences the effect of cholesterol feeding on EDR.

Protocol 3.1

The design of this protocol is shown in Figure 6. At the time of weaning, male New Zealand White rabbits (age 8 - 10 weeks, body weight 1.5 - 2.5 kg) were divided into two batches. The first batch was assigned randomly to control and experimental groups. The control group was given the standard rabbit diet and the experimental group was given the 2% cholesterol diet.

A second batch of animals was continued on the standard rabbit diet for 36 weeks. At this time (age of animals - 46 weeks), the animals were separated randomly into control and experimental groups. The control and experimental animals were given the standard rabbit diet and the 2% cholesterol diet, respectively. The experimental animals, along with their age matched controls were killed for experiments after 4 weeks on their respective diets (the control animals were on standard rabbit diet throughout). The responses to acetylcholine were studied by the tissue bath technique as described previously. At the end of the experiments, the tissues were prepared for histology and sudan red staining.

PROTOCOL FOUR-CALCIUM CHANNEL BLOCKERS AND EDR.

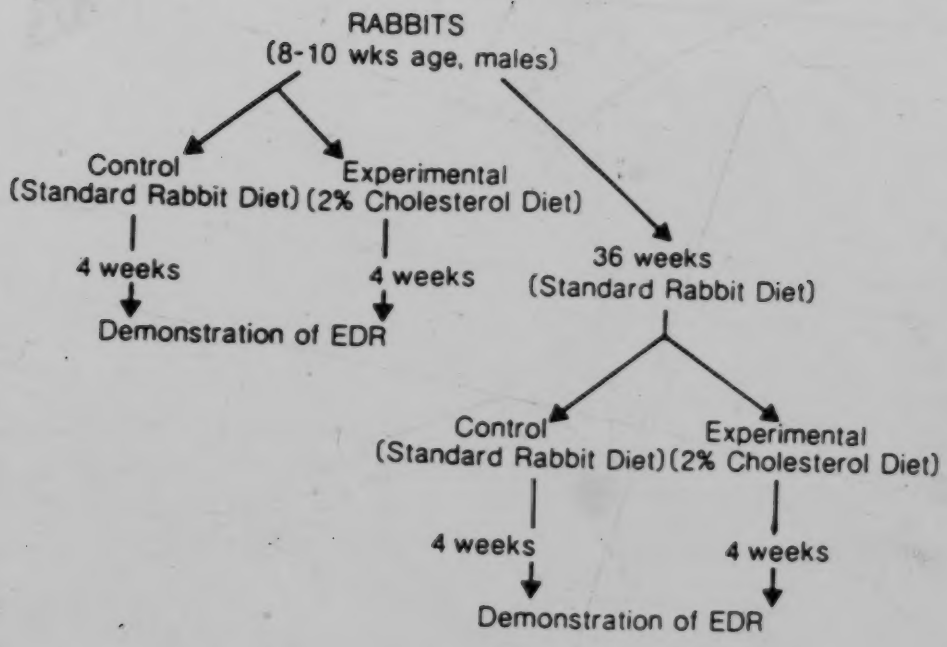

Figure 6. The design of Protocol Three is shown schematically.

The release of EDRF appears to be associated with entry of calcium ions into the endothelial cells. This protocol was designed to investigate the effect of calcium channel blockers on EDR to acetylcholine in the rabbit aorta. The effects of the calcium channel blockers were assessed in tissue baths. In addition, the influence of calcium channel blockers on synthesis/release of EDRF was assessed also. The dihydropyridine derivative, nicardipine, was the principal calcium channel blocker that was used (Satoh *et al.*, 1980). However, in the *in vitro* tissue bath studies the benzothiazepine derivative, diltiazem, was tested also (Ito *et al.*, 1978).

Protocol 4.1.

The effect of chronic oral administration of nicardipine on EDR to acetylcholine was assessed in this protocol.

Administration of nicardipine

Male New Zealand White rabbits of 12 weeks of age were randomized into control and experimental groups. The animals were numbered and housed under similar conditions. Both groups were given the standard rabbit diet. Nicardipine (60 mg/kg) was administered as two divided doses to the experimental group. The pure compound was made into a suspension in water and administered by the oral infusion technique (Moreland, 1965). In this technique, a wooden speculum (15 × 6 × 0.6 cm) with an adequate sized opening was placed transversely in the mouth behind the incisors. The suspension of nicardipine in water was administered via a rubber tube inserted into the oesophagus. After 5 weeks of administration of nicardipine, the animals were killed for experiments. A sample of blood was obtained from the heart for estimation of the serum concentration of nicardipine. The blood was centrifuged at 2000 rpm for 10 minutes and the serum stored at -70°C pending analysis.

Demonstration of EDR

The relaxation responses to acetylcholine were examined in aortic rings isolated from control animals and animals fed nicardipine as described under Protocol 1.1 (i.e.,

tissue bath technique). Aortic rings with and without endothelium were used. All preparations were contracted after the 90 minute equilibration period by adding noradrenaline (-6.0 log mol/l) to the tissue bath. After the contraction had reached a plateau, a concentration-effect curve to acetylcholine was obtained by adding acetylcholine to the tissue bath in a cumulative manner (-9.0 to -4.0 log mol/l). At the end of each experiment, aortic rings were prepared for scanning electron microscopy.

Estimation of nicardipine in serum.

The concentration of nicardipine in serum was estimated by a capillary column gas chromatography technique (Wu *et al.*,1987). The sensitivity of the assay was 0.002 µmol/l. The assay was carried out by Syntex Research, Palo Alto, CA, USA.

Protocol 4.2

The effects on EDR of incubating the rabbit aorta *in vitro* with nicardipine and diltiazem were assessed in this protocol.

Male rabbits, 12 weeks of age, were killed and the descending thoracic aortae removed as described previously. Aortic rings were prepared for tissue bath studies. The endothelium was removed from one ring as described earlier. Each ring was contracted with noradrenaline (-6.0 log mol/l) and concentration-effect curves to acetylcholine were obtained as described before. After the concentration-effect curves to acetylcholine were obtained, the fluid in the tissue baths was replaced with fresh Krebs-bicarbonate buffer and the tension was allowed to return to the baseline value with frequent replacement of the buffer. At this stage, the calcium channel blockers, diltiazem or nicardipine (-5.0 log mol/l) were added to the tissue baths containing an aortic ring with intact endothelium. Another ring with endothelium, with no calcium channel blockers added to the bath fluid served as the control. After 30 minutes of incubation with calcium channel blockers, all the rings were contracted with noradrenaline (-6.0 log mol/l) and the concentration-effect curves to acetylcholine repeated as above . The calcium channel blockers were present in the respective tissue baths throughout the experiment. Only one calcium channel blocker was

tested in one aortic ring. The concentration-effect curves to acetylcholine, in the presence of the calcium channel blockers were compared with the parallel (second) control concentration-effect curve. At the end of the experiment, the aortic rings were prepared for scanning electron microscopy.

Protocol 4.3.

The effects of nicardipine on the synthesis/release of EDRF by the rabbit aorta and on the response to EDRF on the recipient were assessed in this protocol.

The bioassay apparatus shown in Fig 4A was used in these experiments. The aortic tissues were mounted as described in Protocol 2.1. A period of 60 minutes was allowed for equilibration. During the first 30 minutes of this period, a basal tension of 8.0 g was applied on the recipient in a stepwise manner. At the end of 60 minutes, prostaglandin $F_{2\alpha}$ (10 μmol/l) was added to the Krebs-bicarbonate buffer (perfusate from pump P) to produce a contraction in the recipient. When this contraction in the recipient had reached a steady state, the perfusate was supplemented with Krebs-bicarbonate buffer from pump Ac. The pump At was started at the same time and Krebs-bicarbonate buffer containing atropine (10 μmol/l) was added directly onto the recipient. With these two manipulations (i.e., the infusion of buffer proximal to the donor and infusion of atropine on recipient), a slight fall in the contraction to prostaglandin $F_{2\alpha}$ was seen in the recipient due to a dilution effect. When the contraction had stabilized, acetylcholine was infused proximal to the donor at four different concentrations (3.2, 10.0, 31.6 and 100 μmol/l). Each concentration of acetylcholine was infused for 5 minutes. Krebs-bicarbonate buffer was infused for 5 minutes between infusions of acetylcholine. In this way the responses in the recipient to the four concentrations of acetylcholine were obtained.

Over the next 30 minutes, a mixture of atropine (10 μmol/l) and nicardipine (1.0 mmol/l) in Krebs-bicarbonate buffer was infused onto the recipient from pump At. Meanwhile, prostaglandin $F_{2\alpha}$ continued to be infused through the donor and directed onto the recipient. At the end of this 30 minute period, acetylcholine was added again in

the above four concentrations from pump Ac proximal to the donor and the responses in the recipient were determined. During the infusion of acetylcholine, the supplement from At containing atropine (10 μmol/l) and nicardipine (1.0 mmol/l) was continued.

Next, nicardipine (100 μmol/l) dissolved in Krebs-bicarbonate-buffer (containing prostaglandin $F_{2\alpha}$) was infused through the donor via pump P for 30 minutes. At the end of this period, acetylcholine was infused as above and the response in the recipient was determined while maintaining the infusion of nicardipine. In this part of the study, the following sequences of concentration-effect curves relating EDR to acetylcholine were obtained.

1. Control curve with atropine alone on recipient (introduced by pump At).
2. Curve with atropine and nicardipine on recipient (introduced by pump At).
3. Curve with atropine alone on recipient (introduced by pump At) and nicardipine in perfusate from reservoir R. Thus, both the donor and recipient were exposed to nicardipine.

Calculations

The concentration of prostaglandin $F_{2\alpha}$ in the reservoir R was 10 μmol/l. The fluid in the reservoir was diluted 1.304 times by the fluid entering from the infusion pumps (Ac and At) resulting in a final prostaglandin $F_{2\alpha}$ concentration of 7.7 μmol/l at the recipient. The acetylcholine infused (0.45 ml/min) into the perfusate (2.3 ml/min) was diluted approximately 6 times. The concentrations of acetylcholine in the infusion pump were 3.2, 10.0, 31.6 and 100 μmol/l, and after dilution, the concentrations in the fluid perfusing the donor were 0.5, 1.6, 5.3 and 16.6 μmol/l respectively. Atropine infused into the recipient was diluted 12 times. The concentration of atropine in the pump At was 10 μmol/l and after dilution the final concentration on the recipient was 0.83 μmol/l. When nicardipine 1.0 mmol/l was infused on the recipient, after dilution, the final concentration on the recipient was 83.3 μmol/l. Similarly, when nicardipine (100 μmol/l) was infused on the donor,

after dilution, the final concentration on the donor was 76.6 µmol/l. The flow rates from the pumps were kept constant throughout the study.

Protocol 4.4.

The effects of nicardipine and diltiazem on the contractions induced by potassium were assessed in this protocol. This protocol was included to confirm that nicardipine and diltiazem indeed had calcium channel blocking properties under the present experimental conditions.

Aortic rings were obtained from age matched animals and mounted in tissue baths for recording isometric tension. At the end of the 90 minute equilibration period, Krebs-bicarbonate buffer was replaced with a buffer containing 100 mmol/l potassium (see below for composition of the latter buffer). The contraction induced by potassium was allowed to reach a plateau. At this stage the buffer containing 100 mmol/l potassium was removed and replaced with Krebs-bicarbonate buffer. Nicardipine (-5.0 and -4.0 log mol/l) and diltiazem (-4.0 log mol/l) were added to the tissue baths at this stage (only a single concentration of calcium channel blocker was tested in one aortic ring). An aortic ring with no added calcium channel blocker in the tissue bath served as a control. After 30 minutes of incubation with the calcium channel blockers, the buffer containing 100 mmol/l potassium was reintroduced and the response determined in the presence of the calcium channel blockers.

In the experiments in which the potassium induced contractions were studied, the buffer was of the following composition (mmol/l): NaCl 21.0, KCl 100.0, $CaCl_2 \cdot 2H_2O$ 2.5, $NaHCO_3$ 22.0, $NaH_2PO_4.H_20$ 1.2, glucose 10.1, $MgCl_2.6H_2O$ 1.2, $CaNa_2EDTA$ 0.023.

PROTOCOL FIVE-DIABETES AND EDR.

Diabetes mellitus is known to be associated with lesions in the microvasculature and abnormal lipids in the serum. The hypothesis that such lipid abnormalities in the serum and

associated vascular anomalies could interfere with endothelium-dependent vasodilator responses was tested in this protocol.

Protocol 5.1.

The effect of diabetes mellitus on EDR to acetylcholine in rabbit aorta.

Induction of diabetes

Male New Zealand White rabbits, 12-14 weeks of age, were used in the study. The rabbits were made diabetic by injecting alloxan (Bhimji *et al.*, 1985). Alloxan monohydrate (140 mg/kg body weight) was dissolved in normal saline as a 5% solution and injected into the marginal ear vein under local anesthesia (0.5 ml of 2% lignocaine). Those animals who were very restless were sedated with pentobarbitone (15-20 mg/kg) prior to the injection of alloxan. Over the first 48 hours, blood glucose estimations were done frequently. Animals were given 20 ml of 20% dextrose subcutaneously 3-4 times during this period. To prevent dehydration, normal saline (10 ml/kg) was given intravenously 2-3 times over the first 24 to 48 hours also. Three days after alloxan treatment, rabbits were monitored regularly for glycosuria and ketonuria (Keto-Diastix strips, Ames Division, Miles Pharmaceuticals Ltd, Rexdale, Ontario, Canada). Blood glucose estimations were done weekly over the next 12 weeks (Beckman glucose analyser 2, Beckman Instruments Inc, Diagnostic Systems Group, Brea, CA, USA). Blood was collected in capillary tubes (1.1 - 1.2 mm ID, Fisher Scientific) after puncturing the marginal ear vein. Insulin was administered daily as a single dose, subcutaneously. The daily doses of insulin varied between 4-8 units. A mixture of equal parts of soluble insulin (Actapid MC, Connaught NOVO, NOVO INDUSTRI A/S, Denmark) and NPH insulin (NPH Insulin, Connaught NOVO, NOVO Laboratories Ltd, Willowdale, Ontario, Canada) was used. The diabetic animals showed thirst, polyuria, weight loss, glycosuria, ketonuria and elevated blood glucose concentrations. The animals were managed in this manner and sacrificed for experiments at the end of 11 - 12 weeks. Those animals who did not develop the features of diabetes were given a second injection of alloxan (100 mg/kg).

Demonstration of EDR

The rabbits were diabetic for 10 - 12 weeks at the time of killing. Aortic rings were obtained as described perviously and mounted in tissue baths for recording isometric tension. EDR was demonstrated as concentration effect curves to acetylcholine (-9.0 to -4.0 log mol/l). In addition, concentration-effect curves to noradrenaline and methoxamine were obtained also. At the end of the experiments, tissues were prepared for sudan staining and scanning electron microscopy.

STATISTICAL ANALYSIS

In each protocol, the number of rings studied was also the number of rabbits used. The data are expressed as means±standard errors of the mean (s.e. mean). A p value less than 0.05 was considered significant for all analyses. The body weights, the concentrations of serum cholesterol and triglycerides, tissue cholesterol, blood glucose, mean active tensions generated by noradrenaline and the maximal relaxations elicited by acetylcholine were all compared by using the Students t- test for unpaired data. The gradings of sudanophilia in control and cholesterol fed rabbits were compared using the Wilcoxon's rank sum test.

The relaxation responses to acetylcholine were expressed as a percentage of the contraction produced by noradrenaline (-6.0 log mol/l). Concentration-effect curves to acetylcholine in control and experimental animals were compared by analysis of covariance (Snedecor and Cochran, 1980). In this analysis, the concentration of the drug (acetylcholine) was used as the covariate. The cholesterol-fed rabbits and control rabbits were used as two treatment groups with the magnitude of the relaxation as the dependent variable.

Concentration-effect curves to noradrenaline, methoxamine and clonidine were compared by analysis of variance (Cook and Bielkiewicz, 1984). This programme calculated the slopes and ED_{50} values of the concentration-effect curves after probit transformation of the percentage responses. The slopes and ED_{50} values of the

concentration-effect curves from control and experimental groups were then compared by analysis of variance.

RESULTS

In Protocol One, the results will be presented in the following format.

A. General observations on the animals and biochemical data

B. Appearance of the aorta

C. Sudan staining and aortic tissue cholesterol concentrations

D. Light microscopy

E. Scanning Electron Microscopy (SEM)

F. Transmission Electron Microscopy (TEM)

G. Tissue bath data

In the other protocols also, the same format will be followed with appropriate modifications.

PROTOCOL ONE-EFFECT OF CHOLESTEROL FEEDING ON EDR

Protocol 1.1.

Morphology of aorta and EDR to acetylcholine at 0, 4, 8 and 10 week stages of cholesterol feeding.

A. General observations on the animals and biochemical data

At the beginning of the study, the rabbits weighed 2.1 ± 0.2 kg (mean ± s.e. mean, n=6). The control animals on the standard diet gained weight during the study and weighed 3.6 ± 0.5 kg at the end of 10 weeks. The weight gain in the experimental animals on 2% cholesterol diet was less compared to the controls, and at the end of 10 weeks they weighed 2.6 ± 0.1 kg. The body weights of experimental animals given 2% cholesterol diet for 8 and 10 weeks were significantly different from the body weights of the control animals ($p < 0.05$). The body weights, serum cholesterol and triglyceride concentrations in these two groups of animals are summarized in Table 2. The cholesterol and triglycerides concentrations in the serum in experimental animals were significantly elevated compared to the control animals at 4, 8 and 10 week stages of the study. The serum cholesterol

Table 2. Body weight, total serum cholesterol and serum triglycerides in control and experimental rabbits.

0 weeks (commencement of study)	
Body weight (kg)	2.1 ± 0.2
Total cholesterol (mg%)	80.8 ± 5.4
Triglycerides (mg%)	165.5 ± 18.8

Length of study	Control	2% Cholesterol
4 Weeks		
Body weight (kg)	2.6 ± 0.3	2.7 ± . 0.2
Total cholesterol (mg%)	78.7 ± 2.6	2016.0 ± 152.8*
Triglycerides (mg%)	166.8 ± 14.7	313.7 ± 53.7
8 Weeks		
Body weight (kg)	3.7 ± 0.2	2.4 ± 0.2*
Total cholesterol (mg%)	48.0 ± 2.1	1360.0 ± 210.0*
Triglycerides (mg%)	116.8 ± 19.4	414.5 ± 43.1*
10 Weeks		
Body weight (kg)	3.9 ± 0.5	2.6 ± 0.1*
Total cholesterol (mg%)	43.3 ± 3.8	2080.0 ± 103.0*
Triglycerides (mg%)	129.0 ± 26.0	767.0 ± 207.0*
20 Weeks (n=4)		
(i.e., 14 weeks after reversal of diet)		
Body weight (kg)	4.1 ± 0.1	3.9 ± 0.2
Total cholesterol (mg%)	39.0 ± 4.0	50.3 ± 4.0
Triglycerides (mg%)	196.5 ± 56.0	186.0 ± 29.0
38 Weeks (n=5)		
(i.e., 32 weeks after reversal of diet)		
Body weight	4.3 ± 0.2	3.9 ± 0.4
Total cholesterol (mg%)	56.2 ± 8.6	74.2 ± 1.6
Triglycerides (mg%)	114.4 ± 10.3	179.0 ± 35.4

Values are mean ± s.e. mean, n = 6
* Significantly different from control

concentrations showed considerable variation compared to the triglycerides, which showed a persistent upward trend throughout the 10 weeks of cholesterol feeding.

B. Appearance of the aorta

When the experimental animals on the 2% cholesterol diet were killed at 4, 8 and 10 week stages, these animals showed deposition of lipids in a generalized manner. The subcutaneous tissues, liver and the omenta were prominent in this regard. On inspection of the intimal surface of the aorta, yellow coloured spots or streaks were visible. These spots were of different sizes and were most concentrated in the proximal thoracic aorta compared to the distal thoracic aorta and abdominal aorta. Lesions were seen frequently near the origins of the intercostal arteries. At 8 and 10 week stages, larger elevated intimal plaques were seen. In control animals, the aortic intimal surface was smooth and no fatty spots, streaks or plaques were seen.

C. Sudan staining and aortic tissue cholesterol concentrations

In the experimental group, the fatty spots and streaks observed in the aorta, stained red with sudan. The border between stained and unstained area was abrupt. Occasionally, in the proximal thoracic aorta, the entire luminal surface was stained red. An example of the aortic tissue stained with sudan red is shown in Plate 1. Considerable variation was seen in the extent of sudanophilia in the aortae between individual animals. Along with the sudanophilia, the tissue cholesterol concentrations (in the abdominal aorta) were significantly higher in the cholesterol fed animals compared to the controls (Table 3).

In control animals, kept on standard laboratory diet, the luminal surface of the aorta appeared smooth and shiny and most of these specimens did not take up the stain (Plate 1). Rarely, a spot of redness was seen in a few specimens.

D. Light microscopy.

Under the light microscope, the plaques observed in the aortae of experimental animals appeared in transverse section as crescent shaped, elevated structures. These plaques were of different thickness. The larger plaques were as thick as the tunica media

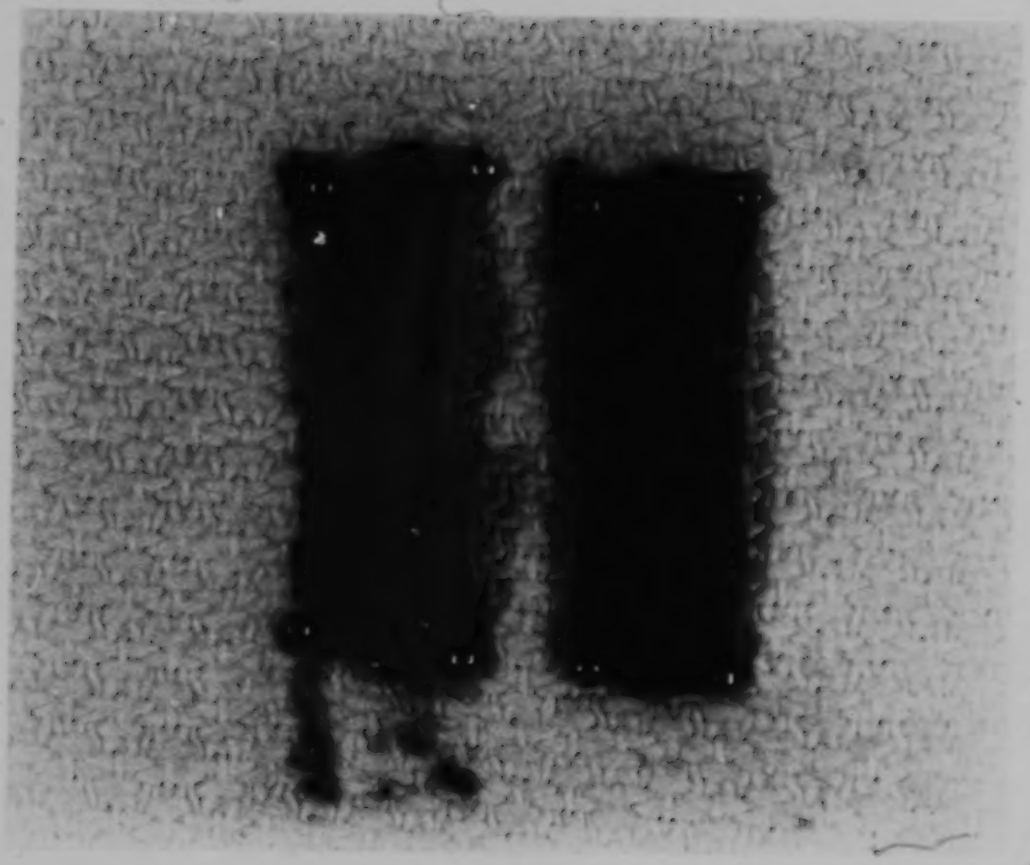

Plate 1. An example of sudan stained specimens from control and atherosclerotic rabbits. Left: control, Right: cholesterol fed (8 weeks stage).

Table 3. Grades of sudanophilia and cholesterol contents of aortae.

Stage of study	No. of animals	Visual grade	Tissue cholesterol content nmol/mg protein
Control	6	0.2 ± 0.1	124 ± 25
Experimental			
4 weeks	6	2.6 ± 0.3	350 ± 62
8 weeks	6	2.4 ± 0.3	604 ± 66
10 weeks	6	2.6 ± 0.3	429 ± 80
20 weeks (reversal)	4	3.6 ± 0.2	1913 ± 430
38 Weeks (reversal)	5	2.8 ± 0.4	2084 ± 360

The visual grades of sudanophilia at 4, 8, 10, 20, and 38 weeks are different from control ($p < 0.05$, Wilcoxon rank sum test). The tissue cholesterol contents at different stages of the study are different from control ($p < 0.05$, t-test for unpaired data).

and the tunica adventitia taken together. In the hematoxylin-eosin stained specimens, the plaques showed numerous vacuoles from which the lipid had been dissolved away. The lipid vacuoles appeared as empty spaces with no apparent limiting membrane. The vacuoles towards the base of the plaque appeared larger than those towards the lumen. The plaques had variable combinations of lipid laden cells and extracellular lipid. The endothelium was seen in some areas while it was absent at other sites. It is likely that this single cell layer got damaged during processing. When lipid stains (osmium tetroxide) were used, the lipid in the plaque was retained and stained black. A transverse section obtained through a plaque is shown in Plate 2. In control animals, the three layers of the aortic wall (tunica intima, tunica media and tunica adventitia) were clearly demarcated. No elevated lesions were seen.

E. Scanning Electron Microscopy (SEM)

Appearance of aorta in cholesterol fed animals

The fatty spots recognized macroscopically and with the light microscope appeared in the SEM as elevated areas. The plaques were oval shaped with well defined or irregular edges. The endothelial cell borders could be traced to these elevations in most areas. In some areas, the endothelial cell borders were indistinct as they approached the elevations. The endothelium was usually intact but in some areas, patchy loss of endothelial cells was seen. The cells appeared swollen and in some areas, thin filamentous projections or short projections with a broad base were seen. Because the cells were swollen and globular, they appeared to be separating from each other. In such areas, multiple strands or bridges running between the cells were noted. Some cells showed holes on their surface and sometimes, the entire surface was covered with one big hole or several small holes. In areas where the endothelium was absent, the fibrillar subendothelial layer was seen underneath. In most of the aortic rings from cholesterol-fed rabbits, approximately 80% of the intimal surface appeared intact. There were no significant differences noted in the appearances of the lesions (i.e., areas of abnormal endothelium, plaques or endothelial

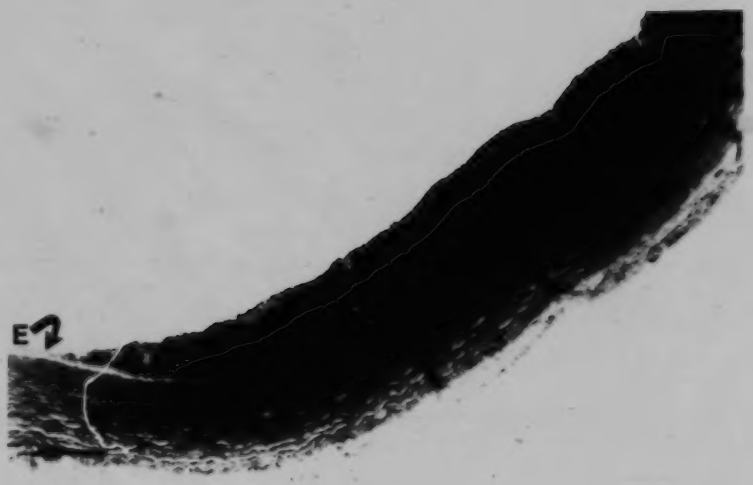

Plate 2. Low power light micrograph showing transverse section through an atherosclerotic plaque in aorta from a cholesterol fed rabbit (osmium tetroxide stain).
M = media, P = plaque, E = endothelium
Magnification × 60, Bar = 1 mm.

ulcers) at 4, 8 and 10 week stages of the study. However, with increasing duration of cholesterol feeding, the frequency of endothelial abnormalities increased. Areas of apparently normal looking endothelium (i.e, the appearances seen in control animals) were seen in cholesterol- fed animals also. The SEM appearances of the aortic luminal surface from cholesterol-fed animals are shown in Plates 3, 4 and 5.

Appearance of the aorta in control animals

In control animals, the endothelial surface was usually uniform and smooth. Longitudinal folds were seen in a few areas. Individual cells were identifiable with clear cell margins in most areas. These cells did not have the swollen appearance or the surface projections that were seen in cholesterol fed animals (Plate 6).

In the aortic rings from which the endothelium was removed at the beginning of the experimental protocol, the fibers of the subendothelial layer were seen. Over 95% of the endothelial cells were missing from these preparations (Plate 7). Isolated islands of cells (less than five cells in each area) were sometimes noted in these deendothelialized rings.

F. Transmission Electron Microscopy (TEM)

Appearance of aorta in cholesterol fed animals

The most striking feature noted in the aortae of cholesterol-fed rabbits was the vacuolation in the cells of the intima. Most endothelial cells had vacuoles of different sizes spread throughout the cytoplasm. The subendothelial layer was widened and contained fragmented elastic fibers. In sections showing plaques, numerous lipid laden foam cells were seen. The lipids were in both intracellular and extracellular locations. The vacuoles towards the base of the plaque were bigger compared to those seen towards the apex. Some of the vacuoles were filled by concentric, lamellar structures. In addition to the lipids, cellular debris were seen in the plaque. The organelles (e.g., mitochondria) in the endothelial cells appeared prominent. The vascular smooth muscle cells in the cholesterol-fed animals also showed vacuolation of the cytoplasm. The TEM appearance through a plaque is shown in Plate 8.

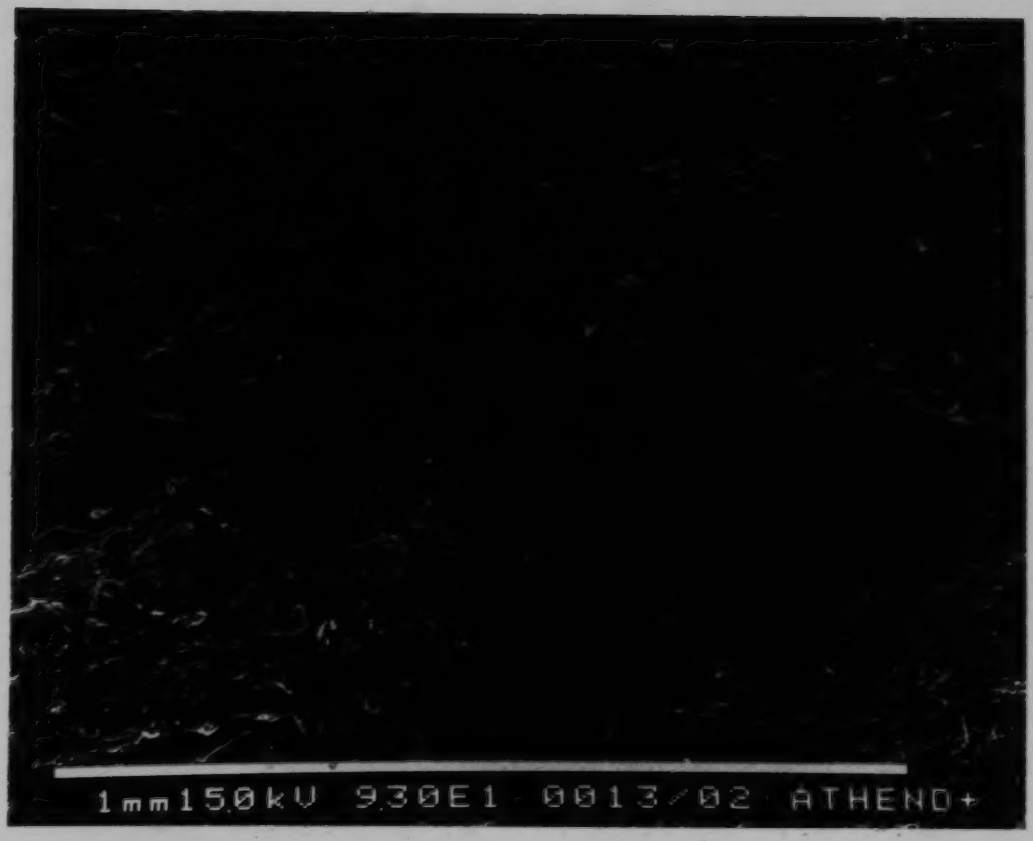

Plate 3. Scanning electron micrograph of the aortic intimal surface in a specimen from a rabbit given the 2% cholesterol diet for 8 weeks. Three plaques can be identified. The black and white bar at the bottom of the picture shows the scale. (bar = 1mm; ×93)

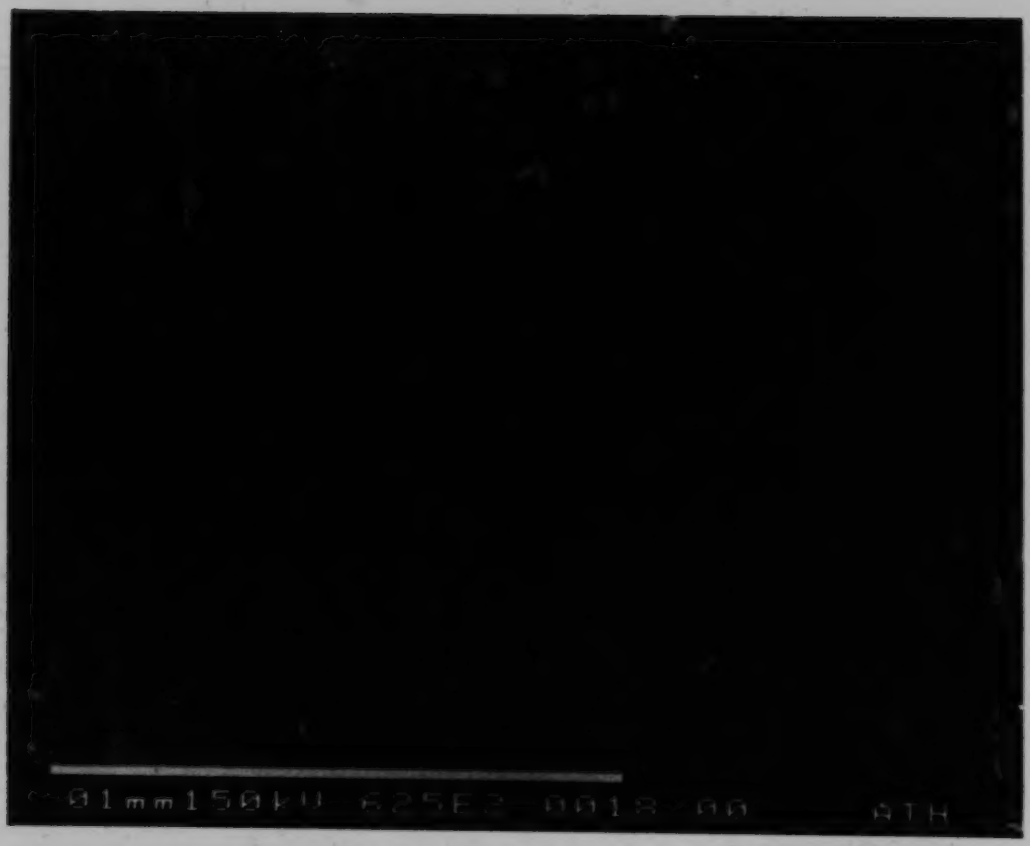

Plate 4. Scanning electron micrograph of the aortic intimal surface in a specimen from a rabbit given the 2% cholesterol diet for 8 weeks. The cells appear swollen and fibrous strands running between cells can be identified. The black and white bar at the bottom of the picture shows the scale. (bar = 0.1 mm; × 625)

Plate 5. Scanning electron micrograph of the aortic intimal surface in a specimen from a rabbit given the 2% cholesterol diet for 8 weeks. Cells with broken cell surfaces can be seen. The black and white bar at bottom of the picture shows the scale. (bar = 0.1 mm; × 625)

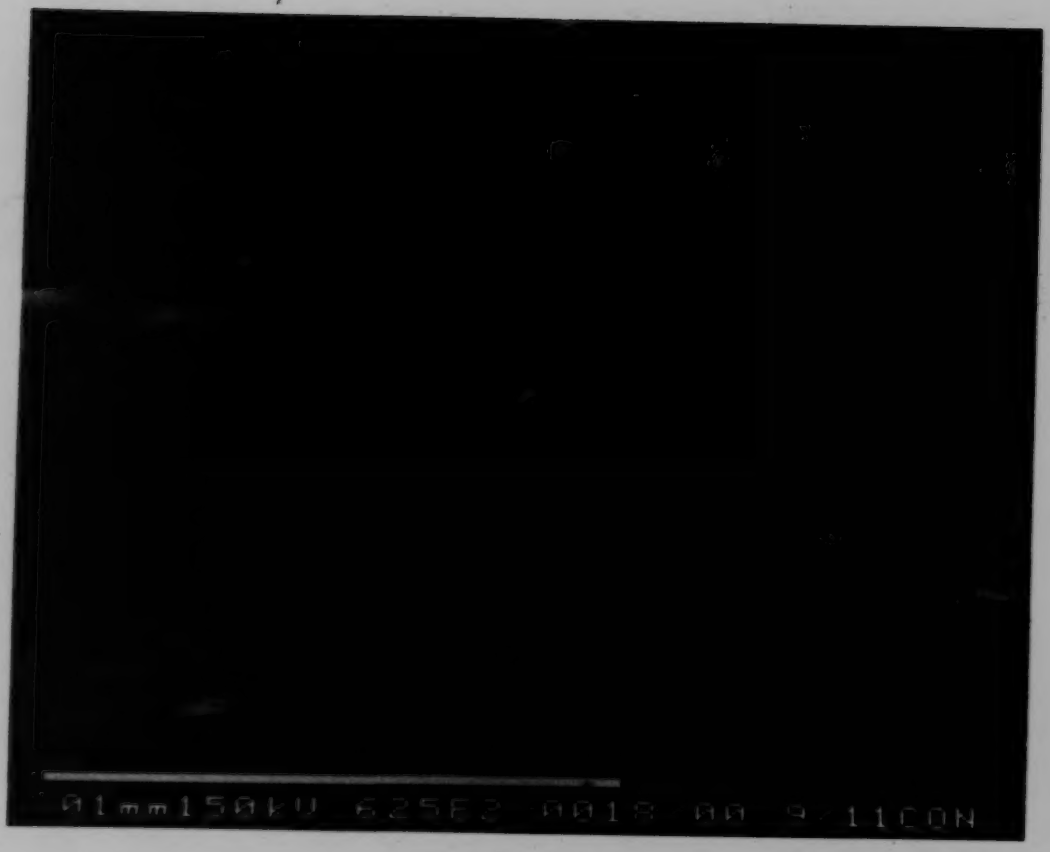

Plate 6. Scanning electron micrograph of the aortic intimal surface in a specimen from a control rabbit showing endothelial cells with clear cell margins. The black and white bar at the bottom of the picture shows the scale. (bar = 0.1 mm; × 625)

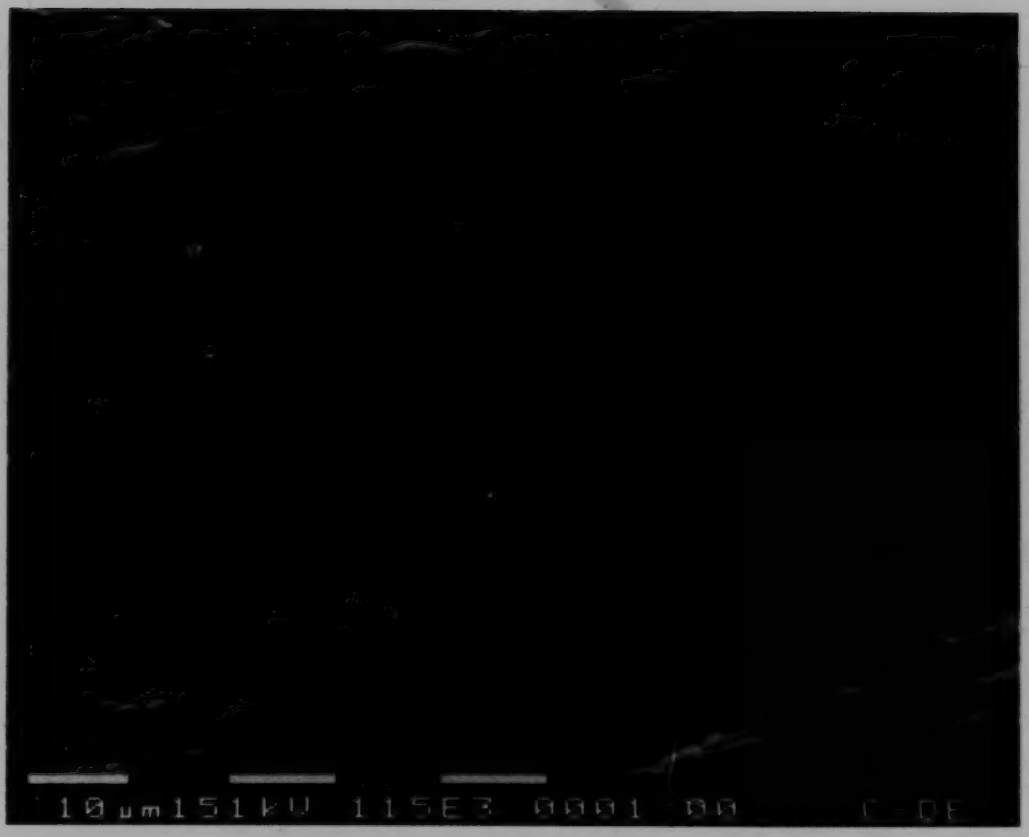

Plate 7. Scanning electron micrograph of the aortic intimal surface in a de-endothelialized specimen from a control rabbit. The fibers in the subendothelial area can be seen. The black and white bar at the bottom of the picture shows the scale. (bar = 10 µm; × 1150)

Plate 8. Transmission electron micrograph of an aortic specimen from a rabbit given the 2% cholesterol diet for 4 weeks. Numerous intracellular and extracellular lipid vacuoles can be seen in the atherosclerotic plaque in the subendothelial area. (bar = 2μm; × 5,600)
EN = endothelial cell, V = vacuoles

Plate 9. Transmission electron micrograph of an aortic specimen from a control rabbit. An endothelial cell can be identified. (bar = 2 μm; × 12,300)
EN = endothelial cell

Appearance of the aorta in control animals

In control animals, the endothelial cell cytoplasm showed numerous vesicles. The subendothelial layer was of uniform thickness and showed elongated smooth muscle cells with branching processes alternating with lamellae of elastic fibers. The TEM appearance of an aortic ring from a control animal is shown in Plate 9.

G. Tissue bath data

Endothelium-dependent relaxation: control state (0 weeks)

When the aortic rings from rabbits were mounted in tissue baths for isometric tension recording, they did not exhibit any spontaneous contractions in the basal state. There was a fall in tension during the equilibration period. The tension was adjusted repeatedly to 8.0 g over the first 30 minutes. The aortic preparations contracted with the addition of noradrenaline (-6.0 log mol/l) to the tissue bath. This contraction reached a plateau in 10-15 minutes. The mean tensions attained are shown in Figure 7.

When acetylcholine was added to the tissue baths containing the pre-contracted aortic rings, the rings with intact endothelium demonstrated a concentration-dependent relaxation. This relaxation response commenced at an acetylcholine concentration of about -8.0 log mol/l and reached a maximum at an acetylcholine concentration of about -6.0 to -5.5 log mol/l. The maximum relaxation to acetylcholine was 48.8 ± 4.5% (n=6, mean ± s.e. mean) of the contractile response to noradrenaline (-6.0 log mol/l). When a lower concentration of noradrenaline (-7.0 log mol/l) was used to pre-contract the vessel, this relaxation to acetylcholine increased to 70.5 ± 3.7%. A similar pattern of results (i.e., a greater relaxation to acetylcholine when a lower concentration of noradrenaline was used to pre-contract the vessel) was seen throughout the study (Table 4).

Indomethacin (-6.0 log mol/l) had no significant effect on this relaxation response produced by acetylcholine. Atropine (-8.0 log mol/l) produced a shift to right of the concentration-effect curve for acetylcholine. When NDGA (-4.4 log mol/l), quinacrine (-6.0 log mol/l) or hydroquinone (-4.0 log mol/l) were added at the point of maximal

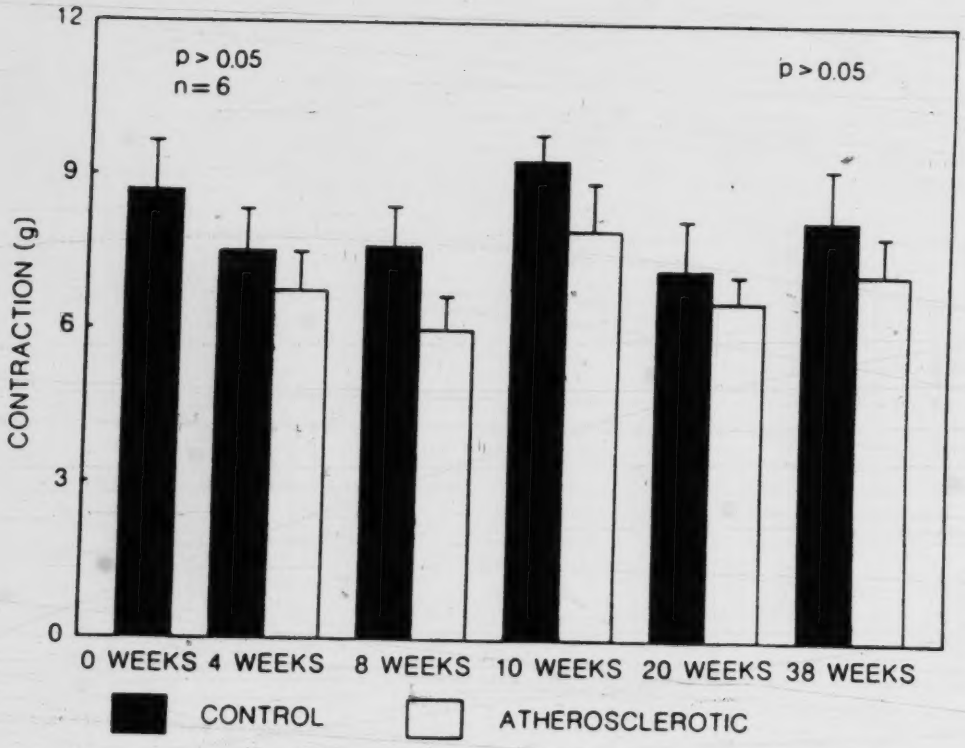

Figure 7. Contractile responses to noradrenaline (-6.0 log mol/l) in aortic rings from control and atherosclerotic animals at 4, 8 and 10 week stages of the study (n = 6). Data for animals in reversal study are shown at 20 weeks (n = 4) and 38 weeks (n = 5). The first group of animals killed at the beginning of the study is shown as 0 weeks. The responses at each stage were not different between control and atherosclerotic animals ($p > 0.05$).

Table 4. Maximum relaxations to acetylcholine (-5.5 log mol/l) following precontraction with noradrenaline in control and experimental animals.

	AT (g)	Relaxation to acetylcholine -6.0 log mol/l NA	-7.0 log mol/l NA
0 weeks (n = 6)			
control	8.8 ± 0.9	48.8 ± 4.5%	70.5 ± 3.7%
4 weeks (n = 6)			
Control	7.5 ± 0.8	45.4 ± 3.6%	56.2 ± 5.8%
Experimental	6.6 ± 0.7	23.2 ± 5.8%	33.0 ± 8.6%
8 weeks (n = 6)			
Control	7.6 ± 0.8	44.5 ± 5.1%	50.6 ± 12.5%
Experimental	6.0 ± 0.6	20.1 ± 8.7%	24.3 ± 5.1%
10 weeks (n=6)			
Control	9.3 ± 0.6	39.0 ± 5.8%	56.0 ± 12.2%
Experimental	7.8 ± 0.9	18.5 ± 8.7%	32.0 ± 7.0%
20 weeks (n = 4)			
Control	7.3 ± 0.9	49.8 ± 11.3%	65.8 ± 12.3%
Experimental	6.6 ± 0.6	20.3 ± 7.1%	32.0 ± 11.0%
38 weeks (n = 5)			
Control	8.2 ± 1.0	34.6 ± 3.9%	-
Experimental	7.2 ± 0.7	14.5 ± 3.8%	-

AT = Active Tension generated by noradrenaline (-6.0 log mol/l)
NA = Noradrenaline
- = response not obtained

relaxation to acetylcholine (-6.0 to -5.5 log mol/l) each caused an almost immediate reversal of this relaxation. The relaxations after adding the three compounds were: NDGA 0.0%; quinacrine 14.9 ± 6.4%; and hydroquinone 0.0% (n=6, $p < 0.05$ for each).

Effect of acetylcholine on de-endothelialized aortic specimens

No relaxation to acetylcholine was seen in rings from which the endothelium was deliberately removed at the commencement of the experimental protocol (maximum relaxation 0.0%, n=6). When the concentration of acetylcholine was increased above -5.0 log mol/l, a contractile response was seen in these rings.

Endothelium-dependent relaxation after cholesterol feeding

Aortic rings from control animals on standard rabbit diet and experimental animals on 2% cholesterol diet showed a contractile response to noradrenaline. The mean tensions generated are shown in Figure 7. Contractions at each stage were not different between control and experimental animals ($p>0.05$). The maximum relaxations to acetylcholine in control animals at 4, 8 and 10 weeks were 45.4 ± 3.6%, 44.5 ± 5.1% and 39.0 ± 5.8% of the contraction produced by noradrenaline, respectively. The corresponding values in the cholesterol fed animals were 23.2 ± 5.8%, 20.1 ± 8.7%, and 18.5 ± 8.7%. Thus, EDR was reduced by at least 50% after cholesterol feeding. Examples of EDR to acetylcholine in aortic rings from control and atherosclerotic animals are shown in Figure 8. The results of the relaxations to acetylcholine and the concentration-effect curves for acetylcholine in the two groups of animals are given in Table 4 and Figure 9 respectively.

In control animals, the maximum relaxations to acetylcholine at 4, 8 and 10 weeks were not significantly different from each other ($p >0.05$). In the experimental animals also, no significant differences were seen in the relaxation responses at the three stages of study ($p >0.05$). The relaxations elicited when -7.0 log mol/l noradrenaline was used to pre-contract the vessels are also given in Table 4.

Indomethacin (-6.0 log mol/l) had no significant effect on the EDR in both control and experimental animals (n=6). Atropine (-8.0 log mol/l) produced a shift to right or

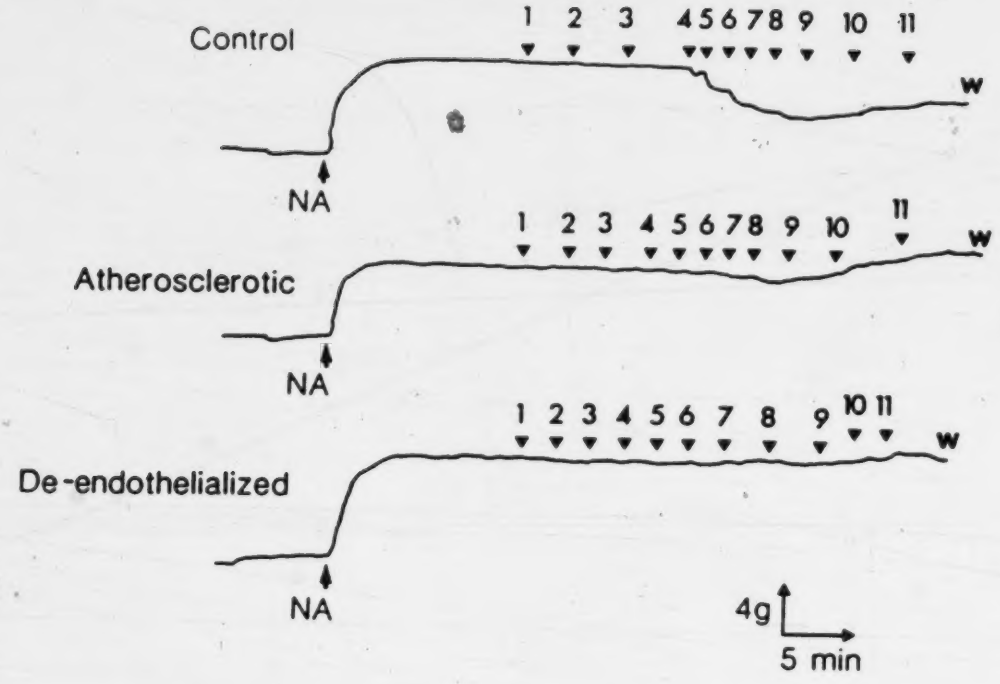

Figure 8. Responses to acetylcholine (-9.0 to -4.0 log mol/l) in aortic rings with endothelium from control and atherosclerotic animals and from de-endothelialized rings. The rings are precontracted with noradrenaline (NA, -6.0 log mol/l) and acetylcholine is added in cumulative manner at points marked 1-11 to give specific concentrations in the tissue bath. The concentrations of acetylcholine (in log mol/l) were as follows: 1. -9.0, 2. -8.5, 3. -8.0, 4. -7.5, 5. -7.0, 6. -6.5, 7. -6.0, 8. -5.5, 9. -5.0, 10. -4.5, 11. -4.0, W = wash.

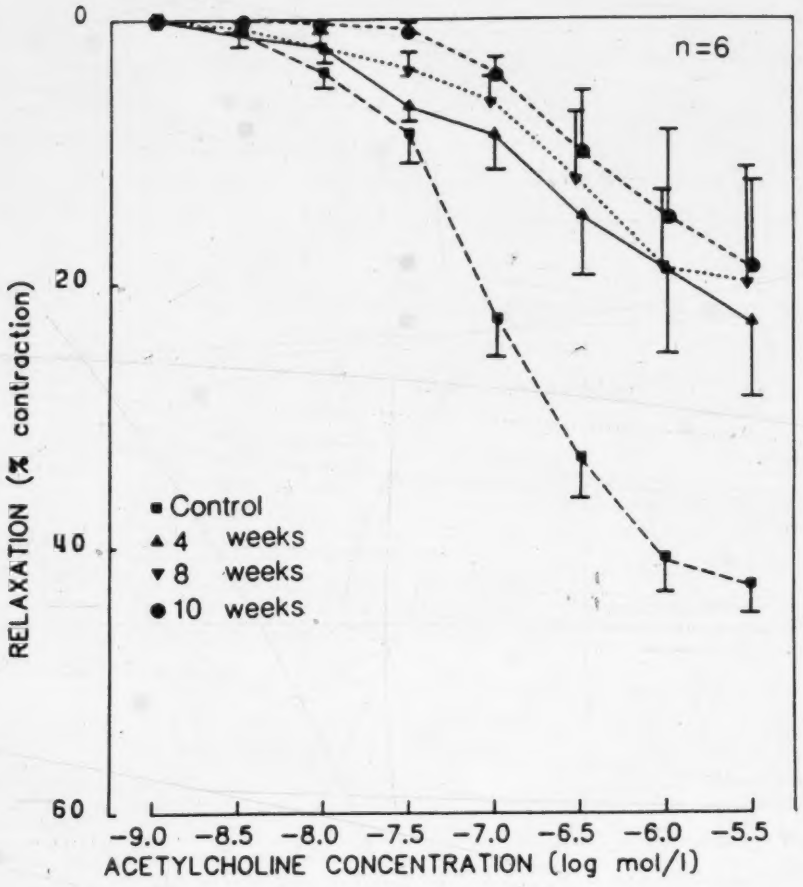

Figure 9. Endothelium-dependent relaxation to acetylcholine during contractions to noradrenaline at different stages of 2% cholesterol feeding. Abscissa shows log mol/l concentration of noradrenaline and the ordinate shows relaxation expressed as percent of contraction of noradrenaline (-6.0 log mol/l). Responses at 4, 8 and 10 weeks of cholesterol feeding are different from control ($p < 0.05$, $n = 6$ for each).

completely inhibited the concentration-effect curve for acetylcholine in both control and experimental animals (n=6). Addition of NDGA inhibited the relaxation produced by acetylcholine. The residual relaxation observed after addition of NDGA was 0.0% (n=6 for each group, $p < 0.05$).

Aortic rings with and without endothelium from control and experimental rabbits showed a relaxation response to sodium nitrite (-4.0 log mol/l and -3.0 log mol/l). No significant difference was seen in this relaxation between the two groups of animals (n=6, $p > 0.05$). These results are summarized in Figure 10. No relaxation to acetylcholine was seen in rings devoid of endothelium from both control and experimental animals at 4, 8 and 10 weeks of study. At concentrations of acetylcholine -5.0 log mol/l and above, a contractile response was seen (n=6 for each group).

Protocol 1.2 (Reversal Protocol)

After an initial 6 weeks of cholesterol feeding, the diets of the rabbits were reversed to standard rabbit diet and the animals were killed after 14 and 32 weeks for demonstration of EDR.

A. General observations on animals and biochemical data

When the animals on the 2% cholesterol diet were reversed to standard rabbit diet, their food intake increased and they gained weight. At the end of 14 weeks on the standard rabbit diet, the reversal animals weighed 3.9 ± 0.2 kg. The age matched control animals who were on standard rabbit diet throughout the study weighed 4.1 ± 0.1 kg. No significant differences were seen in the body weights and serum cholesterol and triglycerides concentrations between the control and reversal animals (Table 2).

B. Appearance of the aorta

Despite being on the standard rabbit diet for 14 and 32 weeks, the animals that had been fed the 2% cholesterol diet still showed deposition of lipids in the viscera at the time of killing. The aorta appeared thickened and the luminal surface was almost completely covered with fatty streaks and plaques.

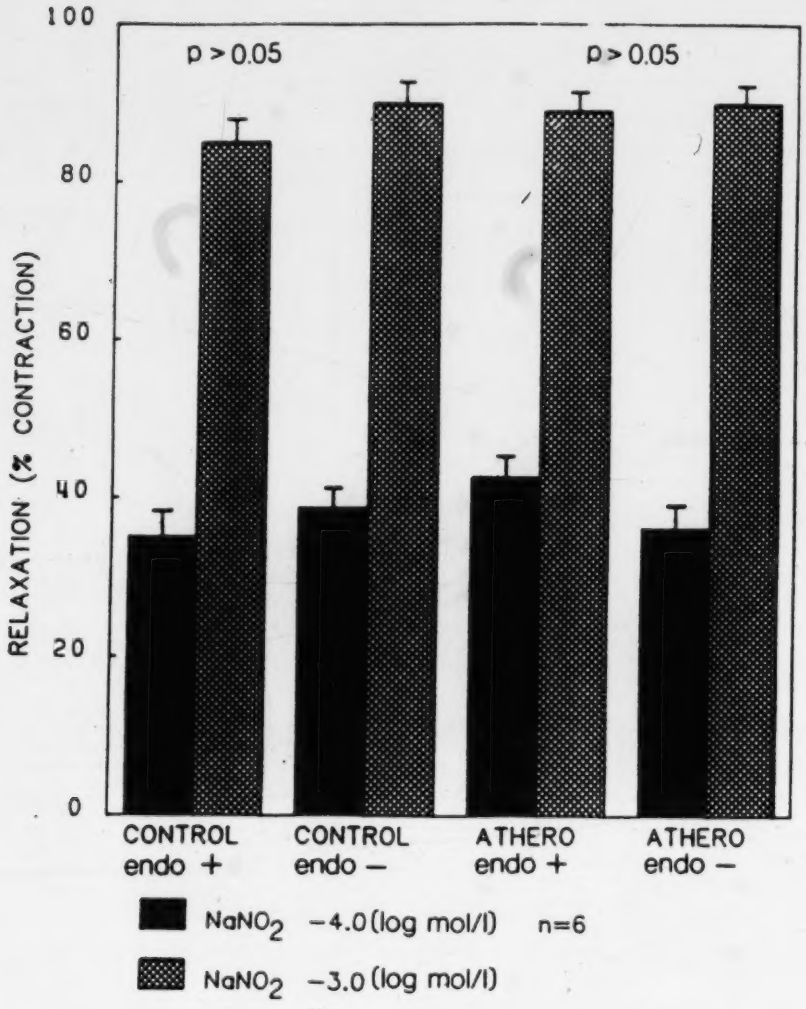

Figure 10. Relaxation responses to sodium nitrite in control and atherosclerotic vessels with and without endothelium. Ordinate shows the relaxation and is expressed as percent of contraction to noradrenaline (-6.0 log mol/l). Response to sodium nitrite (-4.0 log mol/l) in control ring with endothelium (endo +), is compared with responses in atherosclerotic rings with endothelium. Similarly, responses to -3.0 log mol/l in control is compared with response in the atherosclerotic vessel. Responses to sodium nitrite in control and atherosclerotic vessels with and without endothelium are not different from each other ($n = 6$, $p > 0.05$). Data were obtained at the 10 week stage of the study.

C. Sudan staining and aortic tissue cholesterol concentrations

The aortae of experimental animals (reversal group) showed increased sudanophilia. Some specimens were stained with sudan completely. Sudanophilia in these animals was even greater than that seen in animals who were on the 2% cholesterol diet and killed at 4, 8 and 10 weeks (Table 3). The visual grading of sudanophilia in the animals of the reversal study (20 week stage) was significantly more than that of the cholesterol-fed group at the 8 week stage of the study ($p < 0.05$). However, this grading was not significantly different from the grading at 4 and 10 week stages of the study ($p > 0.05$, Table 3).

The tissue cholesterol concentrations were significantly elevated in these animals and this was consistent with the increased sudanophilia. The appearance of the aortae obtained from control animals were similar to the appearances described previously. In the experimental animals, the visual grading of sudanophilia was significantly different from that in the control animals ($p > 0.05$).

D. Scanning Electron Microscopy

SEM of the intimal surface of aortae from the reversal group showed a continuous lining of endothelium in most areas. Cell borders were more distinct and fewer surface projections were seen compared to animals who were still on the 2% cholesterol diet at the time of killing(Plate 10). Also, fewer holes were seen on the cell surfaces in this group of animals.

E. Transmission Electron Microscopy

Under the TEM, the vacuolation of the endothelial and muscle cells were still present. The appearances were similar to those seen in animals who were still on the 2% cholesterol diet at the time of killing (Protocol 1.1).

F. Endothelium-dependent relaxation

Aortic rings from both control and experimental animals contracted to noradrenaline (-6.0 log mol/l) with no significant difference between the active tensions attained (Figure

Plate 10. Scanning electron micrograph of the aortic intimal surface in a specimen from a rabbit given the 2% cholesterol diet for 6 weeks and put back on standard diet for further 14 weeks. The swollen appearance of the cells is less compared to the appearance of cells in animals killed while on the high cholesterol diet (plate 4) and the cell margins can be identified in most areas. (bar = 0.1 mm; × 625)

7). When acetylcholine was added, the aortic rings showed a relaxation response that was maximal at concentrations of -6.0 to -5.5 log mol/l.

The maximum relaxations in the control animals were 49.8 ± 11.3% and 34.6 ± 3.9% (mean ± s.e. mean) of the contraction to noradrenaline at 20 and 38 weeks, respectively. The relaxations in experimental animals were significantly impaired and amounted to 20.3 ± 7.1% and 14.5 ± 3.8% of the contraction, respectively ($p < 0.05$, Table 4). Concentration-effect curves to acetylcholine in control and experimental animals are given in Figures 11 and 12. Thus, the loss of EDR as a result of feeding the 2% cholesterol diet persisted for 32 weeks after the animals were returned to the standard rabbit diet.

Six animals in the group started on the 2% cholesterol diet, died. Three were jaundiced at the time of death and the other three were apparently healthy. Experiments in three others were discontinued due to starvation. No deaths occurred in the reversal and control groups.

Protocol 1.3

The responses of aortic rings from control and experimental animals to α adrenoceptor agonists were studied in this protocol.

Responses to noradrenaline

When studied at 4 weeks, the basal tensions in aortic rings from control and cholesterol-fed animals were 6.0 ± 0.6 g and 5.0 ± 1.0 g respectively (mean ± s.e. mean, n=6). There was no statistically significant difference in the basal tensions in the aortic rings between the two groups of animals ($p > 0.05$). The aortic rings did not exhibit any spontaneous contractions in the basal state. The non-selective α adrenoceptor agonist, noradrenaline (-9.0 to -4.0 log mol/l), produced a concentration dependent contraction. The contractions reached a maximum at concentrations of noradrenaline of -4.5 to -4.0 log mol/l (maximum contraction: 10.9 ± 0.6 g for control, 9.9 ± 1.3 g for cholesterol-fed, mean ± s.e. mean, n=6, $p > 0.05$).

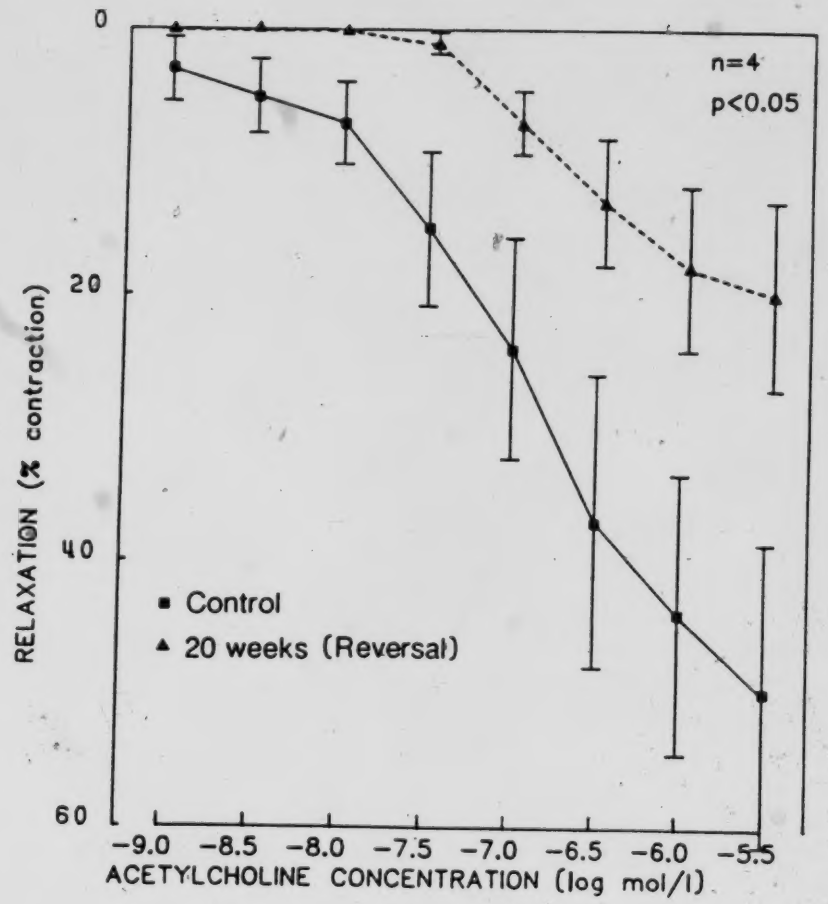

Figure 11. Endothelium-dependent relaxation to acetylcholine during contractions to noradrenaline. Animals in reversal study were put back on standard rabbit diet for 14 weeks after being on 2% cholesterol diet for 6 weeks. Abscissa shows log mol/l concentration of acetylcholine; ordinate shows relaxation expressed as percent of contraction to noradrenaline (-6.0 log mol/l). Responses in the reversal group are significantly different from control ($n = 4$, $p < 0.05$).

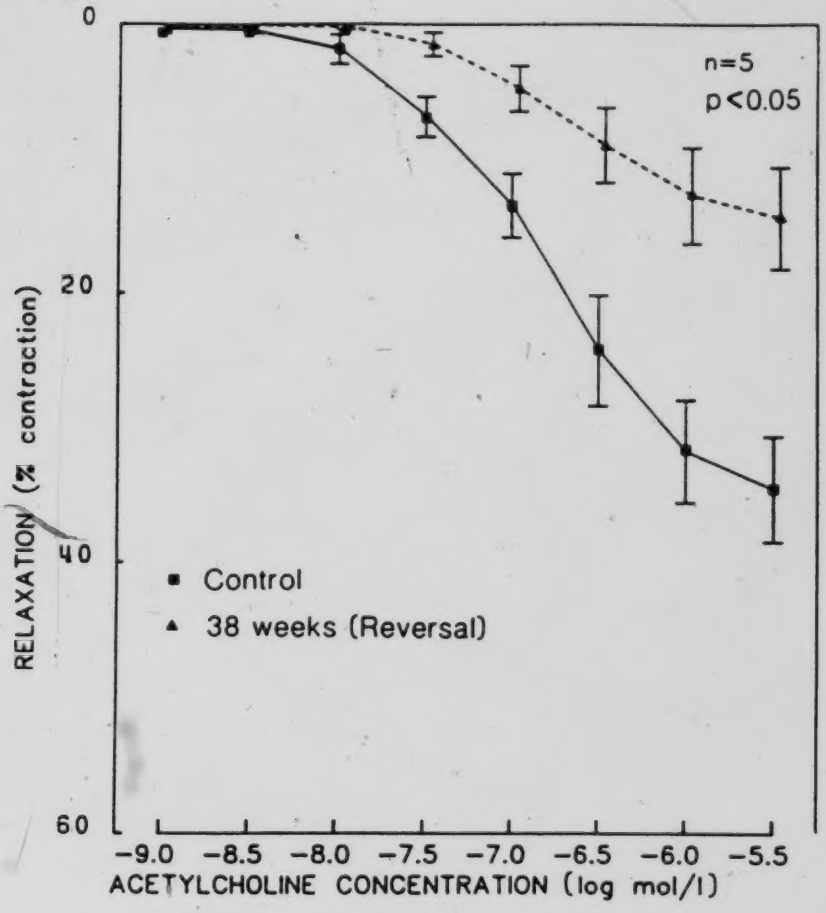

Figure 12. Endothelium-dependent relaxation to acetylcholine during contractions to noradrenaline. Animals in reversal study were put back on standard rabbit diet for 32 weeks after being on 2% cholesterol diet for 6 weeks. Abscissa shows log mol/l concentration of acetylcholine and ordinate shows relaxation expressed as percent of contraction to noradrenaline (-6.0 log mol/l). Responses in reversal group are significantly different from control ($n = 5$, $p < 0.05$).

The ED_{50} values obtained from the concentration effect curves at different stages of the study (4, 8 and 10 weeks: protocol 1.1) are given in Table 5. The slopes and ED_{50} values of the concentration-effect curves between control and cholesterol-fed rabbits were not significantly different from each other ($p > 0.05$). The concentration effect curves obtained for noradrenaline are shown in Figure 13. The only statistically significant finding was an increased contractility (maximum contraction in grams) of aortic rings from atherosclerotic animals at 8 week stage compared to their age matched controls. However, no differences in contractility were seen at 4, 10, 20 and 38 week stages of the study ($p > 0.05$).

Responses to methoxamine

The selective α_1 adrenoceptor agonist methoxamine, (-8.0 to -3.5 log mol/l) produced a concentration-dependent contraction in the rabbit aorta. In comparison with noradrenaline, the contractions elicited by methoxamine developed slowly, taking about 60 minutes to complete a concentration-effect curve. The ED_{50} values obtained from concentration effect curves obtained at different stages of the study are given in Table 6. These values were not significantly different from the corresponding values in the controls ($p > 0.05$).

Responses to clonidine

The effect of clonidine was tested in the rabbit aorta also. Similar to methoxamine, the contractions elicited by clonidine (-8.0 to -4.0 log mol/l) were slow to develop. The responses to clonidine between aortic rings from control and atherosclerotic animals were not significantly different ($p > 0.05$).

SUMMARY

Atherosclerosis was induced in rabbits by feeding a diet supplemented with cholesterol and lipids. The abnormalities noted in the endothelium and subendothelial areas are described. Scanning electron microscopy revealed endothelial cell swelling, cell loss and plaques. When viewed in the transmission electron microscope vacuolation of

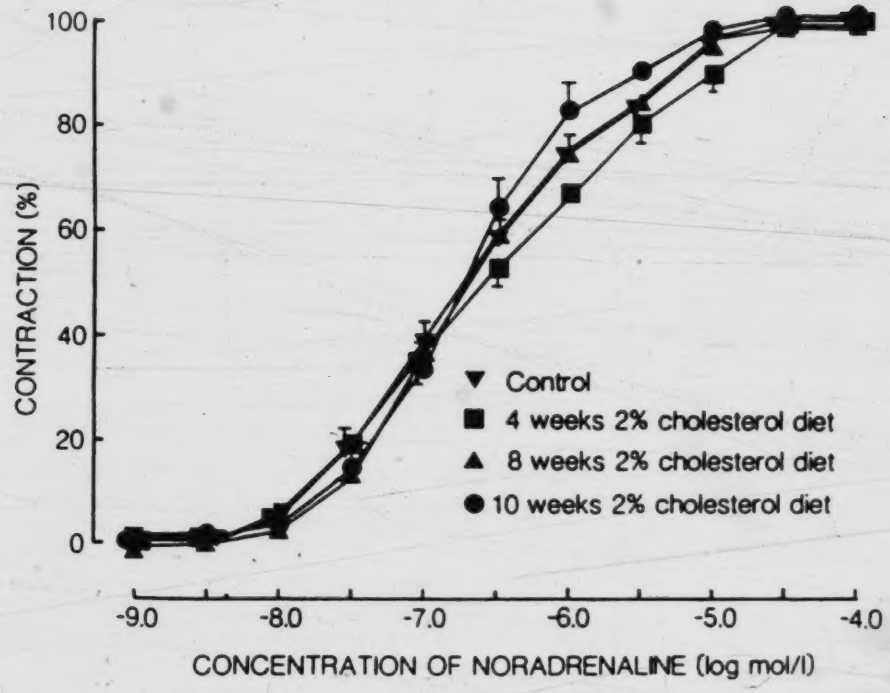

Figure 13. Concentration effect curves for noradrenaline in aortic rings from control and atherosclerotic rabbits. The abscissa shows the concentration of noradrenaline in log mol/l and the ordinate shows the contraction expressed as percent of the maximum contraction. The control refers to data obtained in control animals at 4 week stage of the study. The concentration-effect curves in atherosclerotic animals at 4, 8 and 10 weeks are not significantly different from control ($n = 6$, $p > 0.05$).

Table 5. ED_{50} values for noradrenaline in log mol/l.

Stage of study	Control animals	Experimental animals
0 weeks (n = 6)	-6.69 ± 0.08	-
4 weeks (n = 6)	-6.64 ± 0.08	-6.49 ± 0.10
8 weeks (n = 6)	-6.36 ± 0.15	-6.58 ± 0.09
10 weeks (n = 6)	-6.54 ± 0.11	-6.66 ± 0.14
20 weeks (reversal study)	-6.49 ± 0.15	-6.30 ± 0.06
38 weeks (reversal study)	-6.25 ± 0.20	-6.18 ± 0.01

(n = 4 for 20 week stage and n= 5 for 38 week stage, $p > 0.05$)

Table 6. ED_{50} values for methoxamine in log mol/l.

Stage of study	Control	Experimental
0 weeks (n = 6)	-5.98 ± 0.05	-
4 weeks (n = 6)	-5.77 ± 0.08	-5.98 ± 0.17
8 weeks (n = 6)	-5.85 ± 0.05	-5.91 ± 0.07
10 weeks (n = 6)	-5.97 ± 0.10	-5.80 ± 0.10
20 weeks (reversal study)	-5.95 ± 0.17	-5.98 ± 0.12

(n = 4 for 20 week stage, $p > 0.05$)

cytoplasm of endothelial cells, accumulation of lipids and foam cells in the subendothelial areas were observed. The EDR to acetylcholine was impaired after 4, 8 and 10 weeks on the high cholesterol diet.

When rabbits were put back on standard rabbit diet for 14 and 32 weeks after an initial 6 weeks of 2% cholesterol feeding, the aortae in these animals accumulated cholesterol. The extent of atherosclerosis was significantly higher, compared to animals who were still on the 2% cholesterol diet at the time of killing. The surface abnormalities were less compared to those seen in animals who were still on the 2% cholesterol diet at the time of killing(Protocol 1.1). The EDR to acetylcholine continued to be impaired in the reversal animals compared to controls.

No change in sensitivity to noradrenaline, methoxamine or clonidine were seen in aortae obtained from control rabbits and rabbits given 2% cholesterol diet for 4, 8 and 10 weeks.

PROTOCOL TWO

In this protocol, a bioassay for EDRF was developed. This enabled synthesis/release of EDRF, and the effects of EDRF to be studied separately.

Protocol 2.1

Observations on animals

For developing the bioassay for EDRF, aortae from control rabbits on standard rabbit diet were used.

Bioassay data

Following equilibration, the recipient aortic rings had a mean basal tension of 5.7 ± 0.3 g (mean ± s.e. mean, n=10). When noradrenaline (0.20 µmol/l) was added, the recipient aortic rings demonstrated a contractile response, which reached a plateau in 10-15 minutes. The active tension generated was 3.2 ± 0.2 g, and it could be maintained at a stable level for over two hours.

When the recipient tissue was superfused with Krebs-bicarbonate buffer containing acetylcholine through the donor tissue, a relaxation response was seen within 90-120 seconds. This relaxation response reached a plateau within 5 minutes. Following removal of acetylcholine from the superfusate the tension returned to the baseline value. When increasing concentrations of acetylcholine were introduced into the perfusate, a concentration-dependent relaxation was seen in the recipient (Figure 14). The relaxations obtained with 0.5, 1.6, 5.3 and 16.6 μmol/l of acetylcholine amounted to 16.0 ± 2.7%, 22.3 ± 4.0%, 30.8 ± 5.0% and 32.7 ± 5.3% of the contraction respectively. All the relaxation responses were expressed as a percentage of the contraction obtained by noradrenaline (0.20 μmol/l).

Effect of Atropine

In the bioassay experiments atropine, (0.83 μmol/l) was infused onto the recipient aortic ring separately. When the experiment was performed without infusion of atropine onto the recipient, a pattern of results similar to the above (i.e., concentration dependent relaxation to each dose of acetylcholine) was seen. However, before the relaxation appeared a contractile phase was seen. This contractile response was more prominent with higher concentrations (5.3 and 16.6 μmol/l) of acetylcholine. When atropine was infused onto the recipient separately and the experiment repeated, this contractile phase was abolished and only the relaxation was seen.

Validation of the bioassay technique

Besides acetylcholine, other cholinergic agonists like methacholine (5.0 μmol/l) and carbachol (5.0 μmol/l) and the calcium ionophore (1.5 μmol/l), when infused proximal to the donor, produced a relaxation response in the recipient. This relaxation response in the recipient was evident even when the contraction was produced by prostaglandin $F2\alpha$(10 μmol/l) instead of noradrenaline (n=4).

Incubation of the donor tissue with atropine (0.8 μmol/l) for 30 minutes and removal of the endothelium from the donor tissue abolished the relaxation to acetylcholine

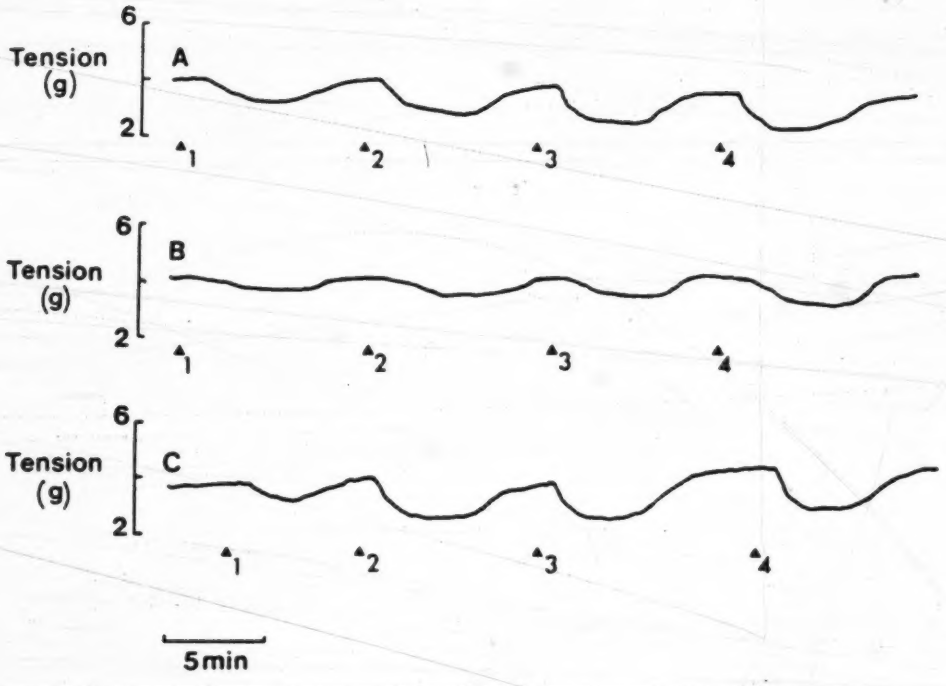

Figure 14. An example of the concentration-dependent relaxation in a recipient ring (obtained from control animals), produced by acetylcholine introduced into the perfusate proximal to the donor aorta. A, response from a control donor (initial control response); B, response from an atherosclerotic donor (test response); C, response from same control donor as in A (final control response). (▲) starting times of the acetylcholine infusion. The infusions were terminated at the nadirs of the relaxations. 1. 0.5 μmol/l; 2. 1.6 μmol/l; 3. 5.3 μmol/l; 4. 16.6 μmol/l.

in the recipient (n=3 for each). Superfusion of the recipient directly with acetylcholine (without prior exposure to the perfusate from the donor aorta) produced no relaxation in the recipient. Infusion of indomethacin (1.0 μmol/l) directly onto the recipient or onto the perfusate proximal to the donor were without effect upon the relaxant response to acetylcholine (n=3).

Protocol 2.2

The synthesis/release of EDRF from control rabbits and rabbits given 2% cholesterol diet for 6 weeks were compared in this protocol.

A. General observations on animals and biochemical data

The control animals on standard rabbit diet and experimental animals on 2% cholesterol diet gained weight on their respective diets and at the end of 6 weeks weighed 3.3 ± 0.1 and 2.6 ± 0.1 kg (mean ± s.e. mean, n=10) respectively. The body weight, serum total cholesterol and triglycerides concentrations in these animals are given in Table 7. At the time of the study, the serum cholesterol and triglycerides concentrations in the animals fed the 2% cholesterol diet were significantly higher than those in control animals. Lipid deposits were seen in the abdominal organs, especially the liver, in these animals.

B. Appearance of donor aortae

Segments of thoracic aorta (4-5 cm in length) obtained from cholesterol-fed and control rabbits were used as donors. Macroscopic examination of the aortae from cholesterol-fed rabbits showed yellow coloured fatty spots and streaks on the intimal surface. These lesions were similar to those described previously (Protocol 1.1). The appearance of the control aorta was similar to those described in protocol 1.1.

Appearance of recipient aortic rings

The recipient tissues were 5 mm rings of aorta obtained from cholesterol-fed or control animals. Fatty spots and streaks were seen on the intima in recipient tissues taken from cholesterol fed animals. When stained with sudan, patches of sudanophilia were seen

Table 7. Effect of feeding diet supplemented with 2% cholesterol for 6 weeks.

	Control	Experimental
Age (weeks)	14 - 16	14 - 16
weight (kg)	3.3 ± 0.1	2.6 ± 0.1*
Total serum cholesterol (mg%)	84.0 ± 10	2109.0 ± 157.0*
Total serum triglycerides (mg%)	128.0 ± 7	751.0 ± 209.0*
Sudanophilia	0.0 ± 0.0	2.1 ± 0.2*
Tissue Cholesterol		
nmol/mg protein	92.0 ± 11	278.0 ± 18.0*
mg/100g wet weight	258.0 ± 36	610.0 ± 47.0*

Values are mean ± s.e. mean, n = 17 for each group
* significantly different from control, $p < 0.05$

in the recipient rings taken from cholesterol-fed rabbits. The recipient tissue from control animals did not show any abnormalities externally and they did not take up sudan stain.

C. Sudan staining

The extent of sudanophilia in the donor aortic segments were significantly greater in the cholesterol-fed animals compared to controls (Table 7). The light and electron microscopic appearances were similar to those described previously (Protocol 1.1).

D. Bioassay data

Experiments using recipients from control animals

Following equilibration for 60 minutes, the aortic rings (recipient tissues) from control animals had a mean basal tension of 5.7 ± 0.3 g (mean ± s.e. mean, n=10). Noradrenaline (0.20 μmol/l) produced a contractile response in these rings.

The mean tensions produced by noradrenaline in the recipient rings were not significantly different during control and test responses (control, 3.2 ± 0.4 g; test, 4.0 ± 0.6 g; $p > 0.05$). When acetylcholine was introduced into the perfusate proximal to either donor (obtained from cholesterol-fed or control animals), a concentration dependent relaxation was observed in the recipient. Examples of such responses obtained by four different concentrations of acetylcholine are shown in Figure 14. It was found that the relaxation responses produced in recipient tissues obtained from control animals by acetylcholine acting on control donors, were significantly greater than those generated from atherosclerotic donors (Figure 15). The maximum relaxations to acetylcholine, obtained from control and atherosclerotic animals were 32.7 ± 5.3% and 16.5 ± 4.9%, respectively (relaxation expressed as a percentage of the contraction to noradrenaline 0.20 μmol/l).

Experiments using recipient from atherosclerotic animals

In the second series of experiments, the recipient tissues were obtained from atherosclerotic animals. The aortic rings had a mean basal tension of 5.0 ± 0.3 g (n=9). The active tension generated in the recipient by noradrenaline (0.20 μmol/l) was 4.1 ± 0.5 g. As in the first series, the relaxation responses to acetylcholine elicited from

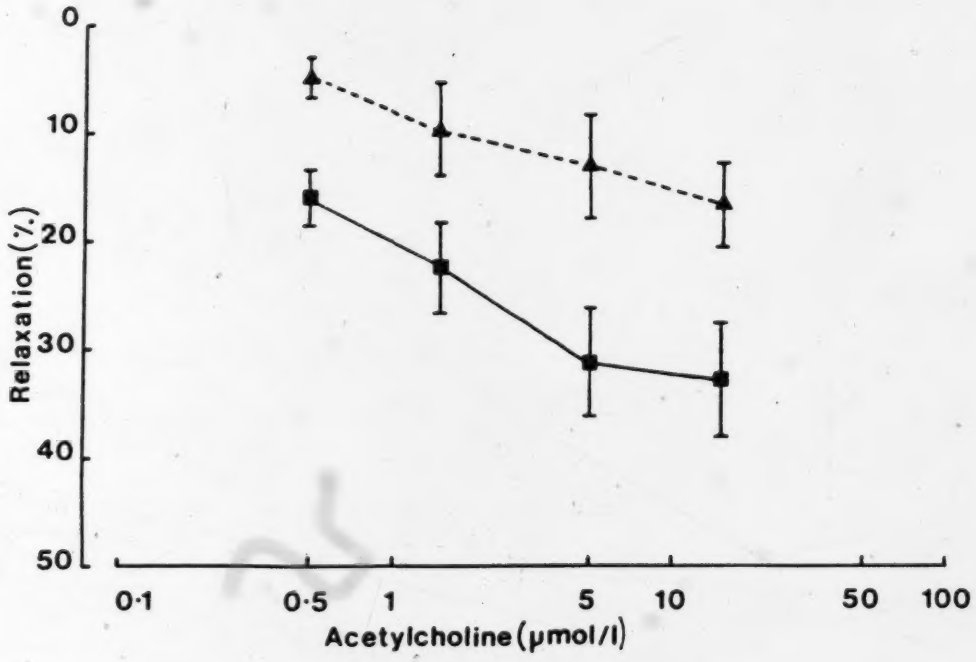

Figure 15. Responses obtained for acetylcholine, when perfused via a control donor and an atherosclerotic donor. Ordinate; relaxation expressed as a percent of the contraction produced by noradrenaline (0.2 μmol/l) on a log scale. ▲ , atherosclerotic animals; ■ , control animals; (n = 10). The responses are significantly different between the two groups of animals ($p < 0.05$).

atherosclerotic donors remained significantly smaller than those generated from control donors ($p < 0.05$). The maximum relaxations to acetylcholine obtained from control and experimental animals were $27.2 \pm 4.4\%$ and $19.8 \pm 4.9\%$ respectively.

Bioassay with a single donor from control animals and different recipients from both control and atherosclerotic animals

The relaxation responses obtained in both control and atherosclerotic recipients when superfused with Krebs-bicarbonate buffer containing acetylcholine passed through control donors, did not differ significantly. The maximum relaxations were $33.2 \pm 5.9\%$ and 27.2 ± 4.4 % for control and atherosclerotic recipients respectively ($p > 0.05$ $n=9$). These results are shown in Figure 16.

Protocol 2.3

The synthesis/release of EDRF in control animals and animals which were put back on standard rabbit diet for 36 weeks after an initial 6 weeks of cholesterol feeding, were compared in this protocol.

A. General observations on animals and biochemical data

At the end of 36 weeks, no significant differences were seen in the body weights or serum cholesterol concentrations between the two groups of animals (Table 8). The liver and retroperitoneal tissues in the experimental animals did not show the severe lipid deposition that was quite prominent in animals who were still on the 2% cholesterol diet at the time of killing (protocol 2.2).

B. Appearance of aorta

The aortae of the experimental animals appeared thickened and were covered with fatty streaks and plaques.

C. Sudan staining

When stained with sudan red, these aortae stained red uniformly (Table 8) . This increased sudanophilia was consistent with the finding of increased tissue cholesterol concentrations in these animals.

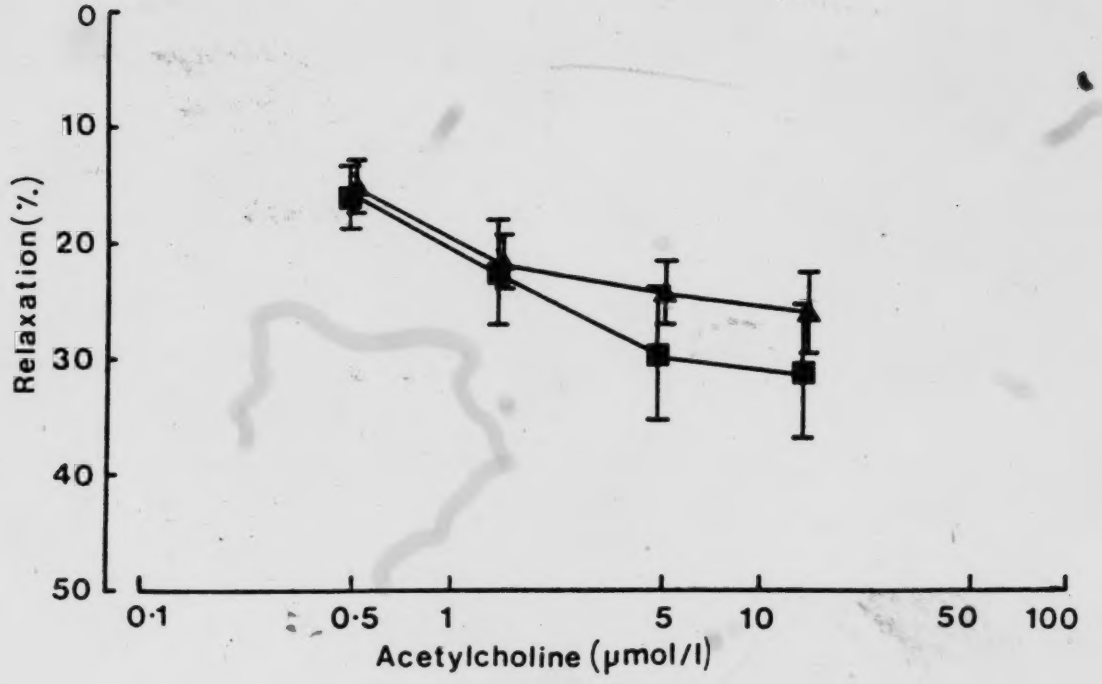

Figure 16. Responses obtained for acetylcholine, when perfused via a control donor. One recipient ring was obtained from a control animal. The other recipient ring was obtained from an animal given 2% cholesterol for 6 weeks. Ordinate: Relaxation expressed as percent of the contraction produced by noradrenaline (0.2 µmol/l). Abscissa: Concentration of acetylcholine (µmol/l) on a log scale. ■ , recipient from control animal; ▲ , recipient from cholesterol fed animal (n = 9). The responses are not significantly different ($p > 0.05$).

Table 8. Effect of reversal to standard rabbit diet for 36 weeks after an initial 6 weeks of 2% cholesterol feeding.

	Control	Experimental
Age (weeks)	52 - 54	52 - 54
Weight (kg)	4.4 ± 0.3	4.5 ± 0.3
Total serum cholesterol (mg%)	43.6 ± 3.3	41.8 ± 4.7
Serum triglycerides (mg%)	115.8 ± 8.4	223.8 ± 43.6
Sudanophilia	0.0 ± 0.0	3.6 ± 0.3*
Tissue cholesterol		
nmol/mg protein	81 ± 6	963 ± 250*
mg/100g wet weight	236 ± 20	2516 ± 730*

Values are means ± s.e. mean, n = 5 for each
* Significantly different from control

D. Light microscopy

Fatty spots and streaks were not seen in these animals. Most of the intimal surface was covered with atherosclerotic plaques of differing thickness. The extent of atherosclerosis was greater in animals where the diets had been reversed.

E. Electron Microscopy

Appearances of experimental cholesterol atherosclerosis similar to those described previously under Protocol 1.2, were seen in these animals. Under the scanning electron microscope, the endothelial cells were found to have a continuous lining and the cell margins were clear in most areas. Elevated plaques were seen. The surface projections were not conspicuous in the aortae from reversal animals. Under the transmission electron microscope, vacuolation of endothelial cells and lipid deposits in the subendothelial areas were seen.

F. Bioassay data

The recipient tissues for the bioassay were obtained from young control rabbits (age 10-12 weeks, body weight 2.3 ± 0.2 kg). Following equilibration, the recipient aortic rings had a mean basal tension of 5.5 ± 0.2 g (mean $\pm$ s.e. mean, n=5). The contractions produced by noradrenaline during control and test responses were 6.0 ± 1.2 g. When acetylcholine was introduced into the perfusate, proximal to the donor (from control or atherosclerotic animals), a concentration dependent relaxation was observed. The responses obtained from control and atherosclerotic donors are shown in Figure 17 As seen in Protocol 2.2, the relaxation responses produced in recipient by acetylcholine acting on control donors were significantly greater than those generated by atherosclerotic donors. The maximum relaxations to acetylcholine observed from control and experimental animals were $36.0 \pm 4.2\%$ and $26.0 \pm 8.5\%$ respectively ($n=5$, $p < 0.05$). As explained under METHODS, the relaxation responses elicited by a common donor, on different recipients could not be obtained. This was due to the poor contractile responses obtained in these rings.

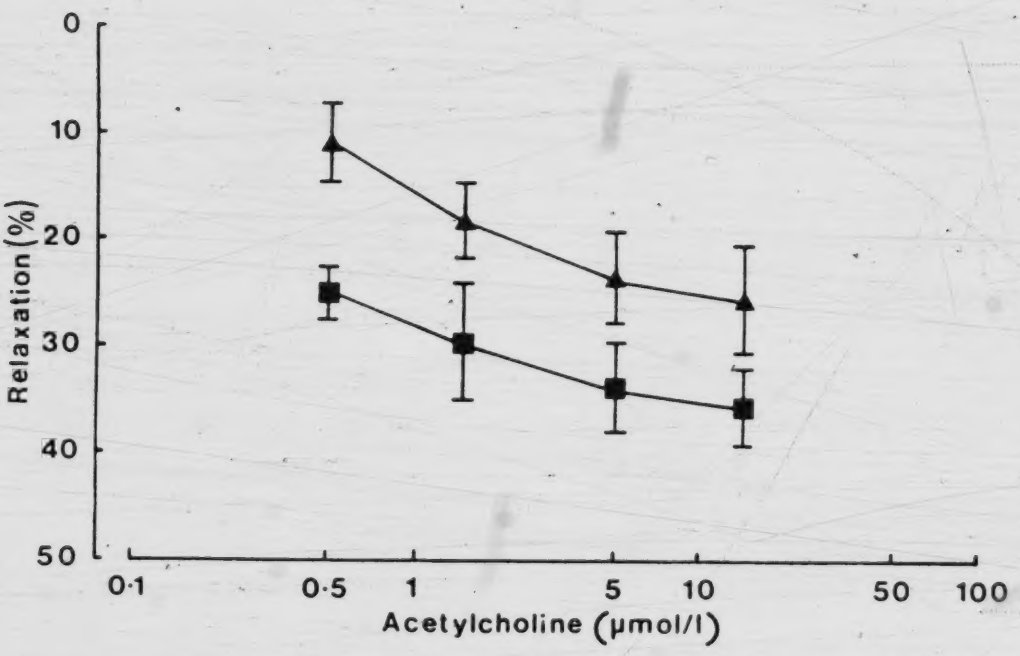

Figure 17. Responses obtained for acetylcholine, when perfused via a donor from a control animal and a donor from a reversal animal. The reversal animals were on standard rabbit diet for 36 weeks after an initial 6 weeks period of high cholesterol diet. Ordinate: Relaxation expressed as percent of the contraction produced by noradrenaline (0.2 µmol/l). Abscissa: Concentration of acetylcholine (µmol/l) on a log scale. ▲ , reversal animals; ■ , control animals (n = 5). The responses are significantly different between the two groups of animals ($p < 0.05$).

Three animals on the 2% cholesterol diet became jaundiced and were discontinued from the study. Two animals showed persistent weight loss and hence were discontinued. No deaths occurred in the control animals and in the animals where diets were reversed.

SUMMARY

A bioassay technique for EDRF was developed. Perfusion of acetylcholine evokes release of EDRF from a segment of donor aorta with intact endothelium. The biological activity of EDRF was monitored on a recipient piece of deendothelialized rabbit aorta. Using this technique, the synthesis/release of EDRF from aortae of control and cholesterol-fed rabbits was compared. The synthesis/release of EDRF was significantly impaired in aortae of atherosclerotic animals compared to control animals. The ability of vascular smooth muscle of the aorta to respond to EDRF appeared to be similar between control and atherosclerotic animals. Synthesis/release of EDRF continued to be impaired in the aortae of animals where the diets were reversed for 36 weeks.

PROTOCOL THREE

The combined influence of age and 2% cholesterol diet on EDR was examined in this protocol.

Protocol 3.1.

12 Week Old Rabbits

A. General observations on animals and biochemical data

At the commencement of the study, this batch of rabbits weighed 2.1 ± 0.2 kg (n=16). The control animals (age: 8 weeks) on the standard rabbit diet, gained weight during the study and weighed 2.9 ± 0.3 kg (n=8, mean ± s.e. mean) at the end of 4 weeks. The experimental animals on 2% cholesterol diet also gained weight at a similar rate and these animals also weighed 2.9 ± 0.2 kg (n=8) at the end of 4 weeks. At the time of killing, these animals were 12 weeks of age. The body weights, serum cholesterol and triglycerides estimations in these animals are summarized in Table 9. Compared to the

Table 9. Body weight, total serum cholesterol and serum triglycerides concentrations in control and experimental rabbits (mean ± s.e. mean). * significantly different from control ($p < 0.05$, t-test for unpaired data).

YOUNG ANIMALS		
Commencement of the study (age 8 weeks, n = 16)		
Body weight (kg)	2.1 ± 0.2	
Total serum cholesterol (mg%)	68.5 ± 5.2	
Serum triglycerides (mg%)	145.5 ± 0.4	
After 4 weeks feeding	Control (n = 8)	2% Cholesterol (n = 8)
Body weight (kg)	2.9 ± 0.3	2.9 ± 0.2
Total serum cholesterol (mg)	76.6 ± 3.1	*1908.3 ± 149.1
Serum triglycerides (mg)	164.6 ± 12.6	*407.3 ± 104.8
OLDER ANIMALS		
Commencement of the study (age 46 weeks, n = 18)		
Body weight (kg)	4.3 ± 0.1	
Total serum cholesterol (mg%)	47.2 ± 2.9	
Serum triglycerides (mg%)	113.9 ± 7.2	
After 4 weeks feeding	Control (n = 9)	2% Cholesterol (n = 9)
Body weight (kg)	4.2 ± 0.1	*2.9 ± 0.1
Total serum cholesterol (mg)	48.7 ± 6.0	*570.7 ± 164.7
Serum triglycerides (mg)	121.0 ± 7.9	*312.0 ± 71.6

control animals, the cholesterol and triglycerides estimations in the serum in experimental animals were significantly elevated ($p < 0.05$).

After 4 weeks on the 2% cholesterol diet, the experimental animals showed deposition of lipids throughout the organs in the body, in particular, the liver.

B. Appearance of aorta

The aortae from the experimental animals showed yellow spots and streaks on the intimal surface. In some animals, confluent yellow lesions covering a large area of the intimal surface were seen. Such lesions were common in the proximal parts of the thoracic aorta compared to the abdominal aorta. No fatty spots or streaks were seen in the aortae from control animals.

C. Sudan staining

Aortic rings from the young animals given the 2% cholesterol diet took up the sudan stain. An occasional aortic ring stained red uniformly. However, considerable variation was seen in the extent of sudanophilia in the specimens from the experimental animals. No sudan staining lesions were seen in the control animals. The extent of sudanophilia was significantly greater in the aortae taken from experimental animals compared to control animals (Table 10, $p<0.05$). The sudan red stained appearances of these blood vessels are shown in Plate 11.

D. Light Microscopy

The appearances of the aortae from the young rabbits given cholesterol were similar to those described previously (Protocol 1.1). Fatty streaks and plaques were seen in the proximal parts of the descending aorta in these animals. Lipid laden foam cells were seen in the plaques. In aortic rings from control animals the thin endothelial lining was seen. The subendothelial layer extending up to the internal elastic lamina was of uniform thickness and had collagen and elastic fibers. Accumulations of lipid laden cells or plaques were not seen in these areas.

E. Scanning Electron Microscopy (SEM)

Table 10. Summary of results from control and 2% cholesterol fed animals of 12 weeks of age. The grades of sudanophilia and the tissue cholesterol content refer to those observed in the rabbit aortic rings. * significantly different from control, $p < 0.05$.

Animal group	Control	Experimental
Age in weeks, number of animals in parentheses	12 (8)	12 (8)
Sudanophilia (visual grade)	0.1 ± 0.1	2.6 ± 0.3*
Tissue cholesterol (nmol/mg protein)	114.2 ± 22.1	417.5 ± 75.4*
Active tension (g) for noradrenaline (-6.0 log mol/l)	7.5 ± 0.8	6.6 ± 0.7
Maximum relaxation to acetylcholine (-5.5 log mol/l)	46.4 ± 2.9%	24.0 ± 4.3%*

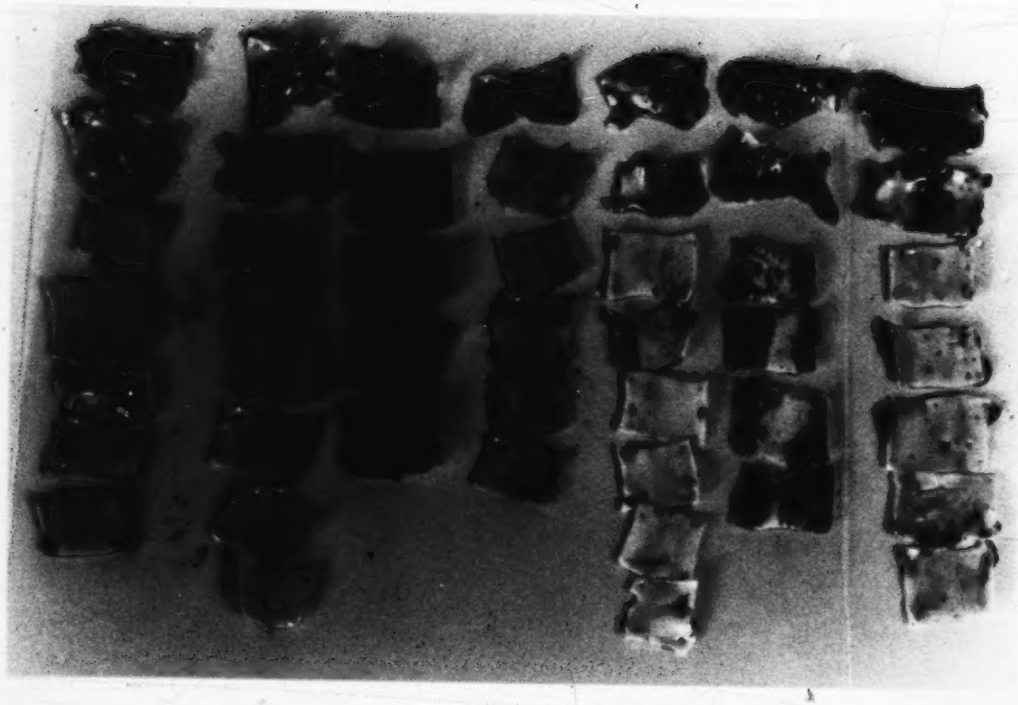

Plate 11. Pieces of rabbit aortic tissue stained with sudan red at the end of tissue bath experiments. These specimens belong to young animals (12 weeks of age) given 2% cholesterol diet for 4 weeks. Sudanophilia was prominent in this group of animals.

SEM of the intimal surface of the aortic rings from experimental animals demonstrated several abnormalities. As described in protocol 1.1, these abnormalities included isolated cell loss, cell swelling and appearance of holes on the cell surface. Some areas of apparently normal looking endothelium (i.e., similar to those seen in control animals) were observed in cholesterol-fed animals also.

F. Transmission Electron Microscopy (TEM)

In the experimental animals, the endothelial cells showed vacuoles of different sizes spread throughout its cytoplasm. The subendothelial layer was not of uniform thickness and demonstrated foam cells. Lipid deposition was found inside these foam cells and in extracellular locations. The vacuoles towards the base of the plaque appeared larger than those at the apex. The majority of the vacuoles were circular and did not appear to be limited by a membrane. Most of these vacuoles were empty, while a few contained concentrically arranged irregular structures. Vacuoles were seen interspersed between the smooth muscle cells of the tunica media also.

In control animals, the endothelial cells formed a continuous layer. The cytoplasm of these cells contained numerous vesicles. The subendothelial layer was of uniform thickness and showed elongated smooth muscle cells with branching processes and lamellae of elastic fibers in between these cells. The TEM appearances of the aortae in these animals are similar to that shown in Plate 8.

G. Tissue bath data

Aortic rings from control and experimental animals were studied in tissue baths. Preparations from both control and experimental animals contracted with the addition of noradrenaline, reaching a plateau within 10-15 minutes. The mean tensions attained in the rings from control and experimental groups were not significantly different from each other ($n=8$, $p > 0.05$). These results are shown in Table 10.

When acetylcholine was added, the control rings demonstrated a concentration-dependent relaxation. This relaxation commenced at an acetylcholine concentration of -8.0

log mol/l and reached a maximum about -6.0 to -5.5 log mol/l. A further increase in the concentration of acetylcholine led to a reversal of the relaxation response. The maximum relaxation to acetylcholine in control preparations was 46.4 ± 2.9% (mean ± s.e. mean) of the contractile response to -6.0 log mol/l noradrenaline (Figure 18). Indomethacin (-6.0 log mol/l) had no significant inhibitory effect on this relaxation response to acetylcholine. Atropine (-8.0 log mol/l) produced a parallel shift to right or complete inhibition of the concentration-effect curve to acetylcholine. No relaxations to acetylcholine were seen in aortic rings in which the endothelium was deliberately removed at the commencement of the experimental protocol.

The aortic rings from the experimental animals demonstrated an endothelium-dependent relaxation to acetylcholine. However, the relaxant responses to acetylcholine in the experimental group were significantly less than that observed in the control group (n=8, $p < 0.05$, Figure 18). The maximum relaxation was 24.0 ± 4.3% (mean ± s.e. mean) of the contractile response to noradrenaline (-6.0 log mol/l).

50 Week Old Rabbits

A. General observations on animals and biochemical data

At the beginning of 46 weeks, the rabbits (before being randomized to control and experimental groups) weighed 4.3 ± 0.1 kg. When the experimental group of rabbits were commenced on a diet containing 2% cholesterol at 46 weeks of age, their food intake decreased considerably, resulting in loss of weight. At the end of the 4 weeks the weight of the rabbits in the experimental group was 2.9 ± 0.1 kg (n=9, mean ± s.e. mean). The weight of the control group on standard rabbit diet remained stable. Their weight at the end of 4 weeks was 4.2 ± 0.1 kg, (n=9, mean ± s.e. mean). The body weight in the control group was significantly greater compared to the experimental group at the end of feeding the respective diets for 4 weeks ($p < 0.05$, Table 9). The cholesterol and triglycerides concentrations in the serum in experimental animals were significantly elevated compared to the control animals (Table 9, $p < 0.05$). However, the serum cholesterol concentration in

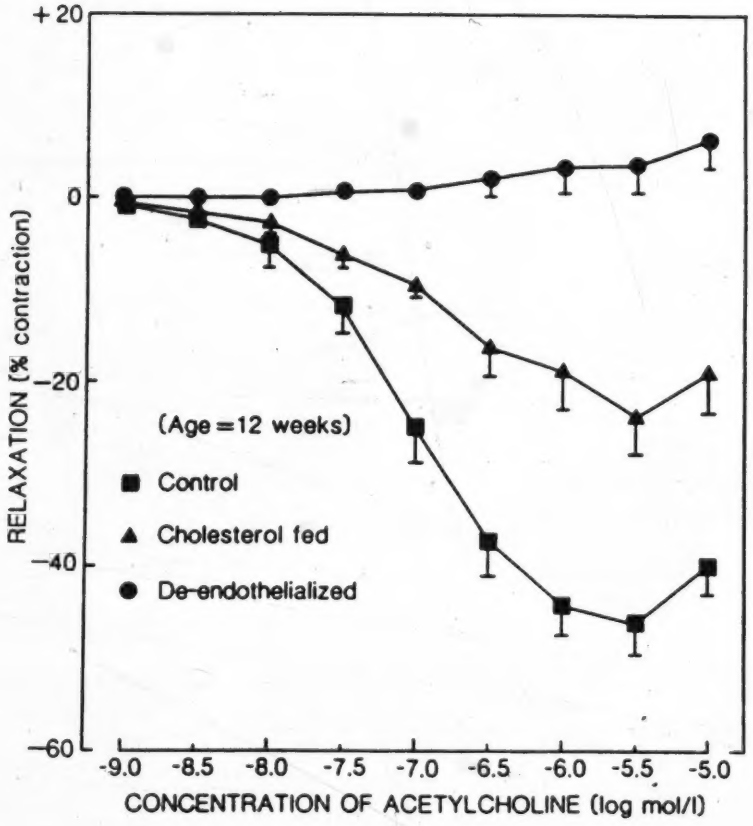

Figure 18. Endothelium-dependent relaxations to acetylcholine during contractions to noradrenaline in young rabbits (8 weeks of age) given 2% cholesterol diet for 4 weeks. Abscissa shows log mol/l concentration of acetylcholine and the ordinate shows the relaxation expressed as percent of contraction to noradrenaline(-6.0 log mol/l). The responses between control and atherosclerotic animals are significantly different from each other (n= 8, $p < 0.05$). The rings without endothelium showed a contractile response at higher concentrations of acetylcholine. -: Relaxation; +: Contraction.

the rabbits fed a 2% cholesterol diet commencing at 46 weeks of age was significantly less than that in rabbits fed this same diet commencing at 8 weeks of age for 4 weeks ($p < 0.05$).

After 4 weeks of feeding with a 2% cholesterol diet, the experimental group of animals showed evidence of deposition of lipids in the liver. However, the amount of lipids in the subcutaneous tissue, mesentery and the retroperitoneal tissue appeared to be less than that observed in the younger experimental animals (no quantitative data are available).

B. Appearance of aorta

The aortae from 8 out of 9 older experimental animals did not show any evidence of fatty streaks or plaques. In one animal, fatty spots and streaks were seen.

C. Sudan staining

In aortae from 8 out of 9 experimental animals, no evidence of sudanophilia was seen. The single animal showing fatty spots and streaks showed positive sudan staining of the aorta. The extent of sudanophilia in the aorta in the experimental group was not significantly different from the controls (Table ll, $p > 0.05$). The sudan red stained appearances of these aortae are shown in Plate 12.

D. Light microscopy

Under the light microscope, the appearances of the aortic rings from experimental animals were similar to those of control animals. Except for one animal, lesions of atherosclerosis were not seen in these animals. Atherosclerotic plaques containing lipid laden cells were not seen in the subendothelial areas.

E. Scanning Electron Microscopy

The SEM appearances of the aortic intima in these experimental animals (50 weeks of age) were similar to the appearances seen in the young experimental animals. Cell swelling, loss of isolated cells, loss of cell margins and cells with surface projections were seen. In the control animals the endothelium formed a continuous lining and cells with clear margins were seen.

Table 11. Summary of results from the control and 2% cholesterol fed animals of 50 weeks of age. The grades of sudanophilia and the tissue cholesterol content refer to those observed in the rabbit aortic rings. * significantly different from control, $p < 0.05$.

Animal group	Control	Experimental
Age in weeks, number of animals in parentheses	50 (9)	50 (9)
Sudanophilia (visual grade)	0.0 ± 0.0	0.2 ± 0.2
Tissue cholesterol (nmol/mg protein)	88.9 ± 11.4	181.2 ± 29.7*
Active tension (g) for noradrenaline (-6.0 log mol/l)	8.9 ± 0.7	8.1 ± 0.4
Maximum relaxation to acetylcholine (-5.5 log mol/l)	31.8 ± 3.9%	9.1 ± 1.5%*

Plate 12. Pieces of rabbit aortic tissue stained with sudan red at the end of tissue bath experiments. These specimens belong to older rabbits (50 weeks of age) given 2% cholesterol diet for 4 weeks. Only one of these animals showed sudan staining.

F. Transmission Electron Microscopy

The experimental animals of 50 weeks of age did not show the prominent changes of atherosclerosis which were seen in the younger (12 week) experimental animals. Although the endothelial cells showed abnormal vacuolation, the subendothelial changes were less conspicuous compared to those seen in younger experimental animals. Very few lipid laden cells were seen in the subendothelial layers of the aortae of these animals. Appearances in aortae from control animals were similar to those described previously. The TEM appearance of the intima and the adjacent media of the aorta from a cholesterol-fed animal is shown in Plate 13.

G. Tissue bath data

Preparations from both control and experimental groups contracted with the addition of noradrenaline (-6.0 log mol/l). The mean tensions attained in the two groups were not significantly different from each other (Table ll, $p > 0.05$). When acetylcholine was added, the control rings demonstrated a concentration-dependent relaxation similar to that observed in the younger control animals. The maximum relaxation observed was 31.8 ± 3.9% (mean ± s.e. mean) of the contractile response to -6.0 log mol/l of noradrenaline (n=9, Figure 19). No relaxation to acetylcholine was seen in deendothelialized rings.

The relaxation response to acetylcholine in the experimental group was less than that observed in the control group (n=9, $p < 0.05$, Figure 19). The maximum relaxation observed was 9.1 ± 1.5% of the contractile response to noradrenaline.

SUMMARY

Both young and older rabbits fed on atherogenic diet showed an impairment of EDR to acetylcholine. The extent of atherosclerosis was significantly less in the older animals compared to the young animals. SEM appearances of the aortic endothelium were similar in both age groups. However, the TEM appearances of classical atherosclerosis (subendothelial plaques) were seen only on young animals. In the older animals, only

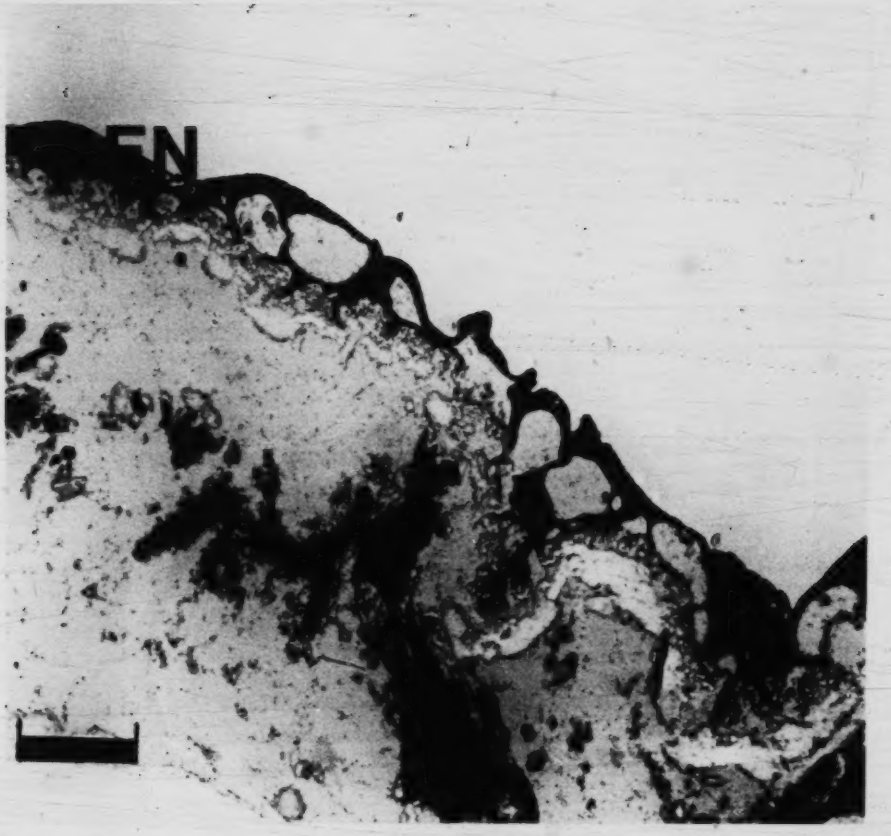

Plate 13. Transmission electron micrograph of an aortic specimen from an older rabbit (50 weeks of age) given the 2% cholesterol diet for 4 weeks. Prominent vacuoles can be seen in the endothelial cells. In contrast to Plate 8, no subendothelial lipid vacuoles are seen (bar = 2 μm).
EN = endothelial cell

× 7,200

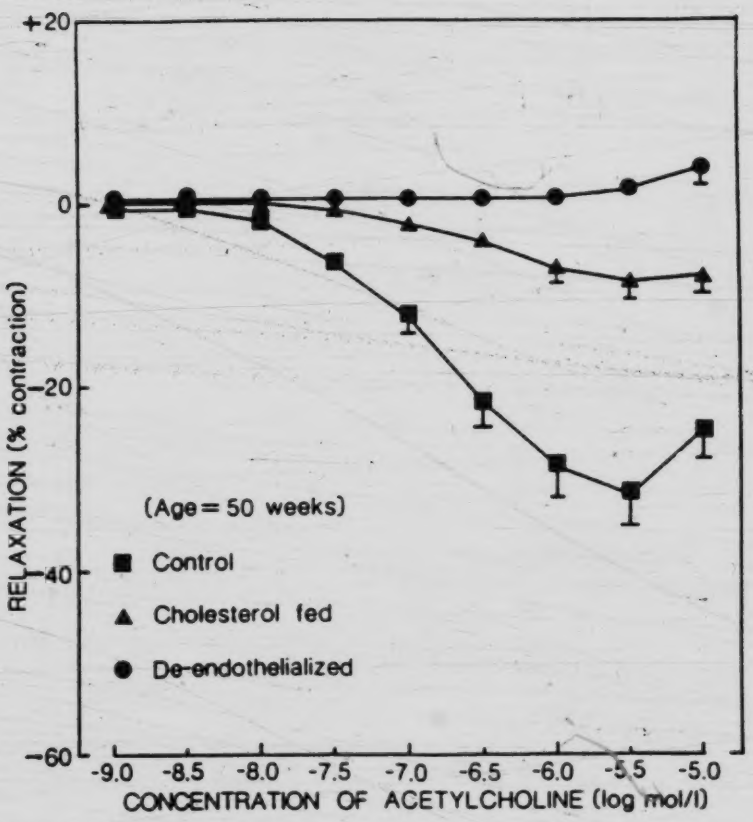

Figure 19. Endothelium-dependent relaxations to acetylcholine during contractions to noradrenaline in old rabbits (46 weeks of age) given 2% cholesterol diet for 4 weeks. Abscissa shows log mol/l concentration of acetylcholine and the ordinate shows the relaxation expressed as percent of the contraction to noradrenaline (-6.0 log mol/l). The responses between control and atherosclerotic animals are significantly different ($n = 9$, $p < 0.05$). The rings without endothelium showed a contractile response at higher concentrations of acetylcholine. -: Relaxation, +: Contraction.

endothelial abnormalities were seen. In addition, a significant weight loss was seen in the older animals.

PROTOCOL FOUR

The effects of calcium channel blockers, nicardipine and diltiazem on EDR to acetylcholine were investigated in this protocol. Control rabbits on standard rabbit diet were used throughout this protocol.

Protocol 4.1

The effect of chronic oral administration of nicardipine on EDR to acetylcholine.

A. General observations on animals and biochemical data

At the commencement of the study, the rabbits weighed 2.1 ± 0.2 kg (n=12). Both control animals and animals fed nicardipine 60 mg/kg/day for 5 weeks gained weight on the standard rabbit diet over the period of study and at the time they were killed, weighed 3.2 ± 0.2 kg (n=6) and 3.1 ± 0.6 kg (n=6) respectively. The gain in weight in control and nicardipine-fed animals were not significantly different from each other ($p>0.05$).

B. Tissue bath data

The aortic rings from both control and nicardipine-fed groups did not exhibit any spontaneous contractions in the basal state. Preparations from both groups of animals contracted with the addition of noradrenaline (-6.0 log mol/l) reaching a plateau within 10-15 minutes. The mean tensions attained in control and nicardipine-fed animals were 7.7 ± 0.9 g and 7.0 ± 0.5 g respectively (mean ± s.e. mean, n=6, $p>0.05$). When acetylcholine was added, aortic rings from both groups demonstrated a concentration dependent relaxation. This relaxation commenced at an acetylcholine concentration of approximately -8.0 log mol/l and reached a maximum at an acetylcholine concentration of -6.0 to -5.5 log mol/l. The maximum relaxations to acetylcholine in aortic rings from control and nicardipine fed animals were 43.6 ± 5.5% and 53.8 ± 6.7% (mean ± s.e. mean) of the contractile response to noradrenaline (n=6). Hence, nicardipine had no significant inhibitory effect on EDR to acetylcholine ($p>0.05$). The concentration-effect curves to

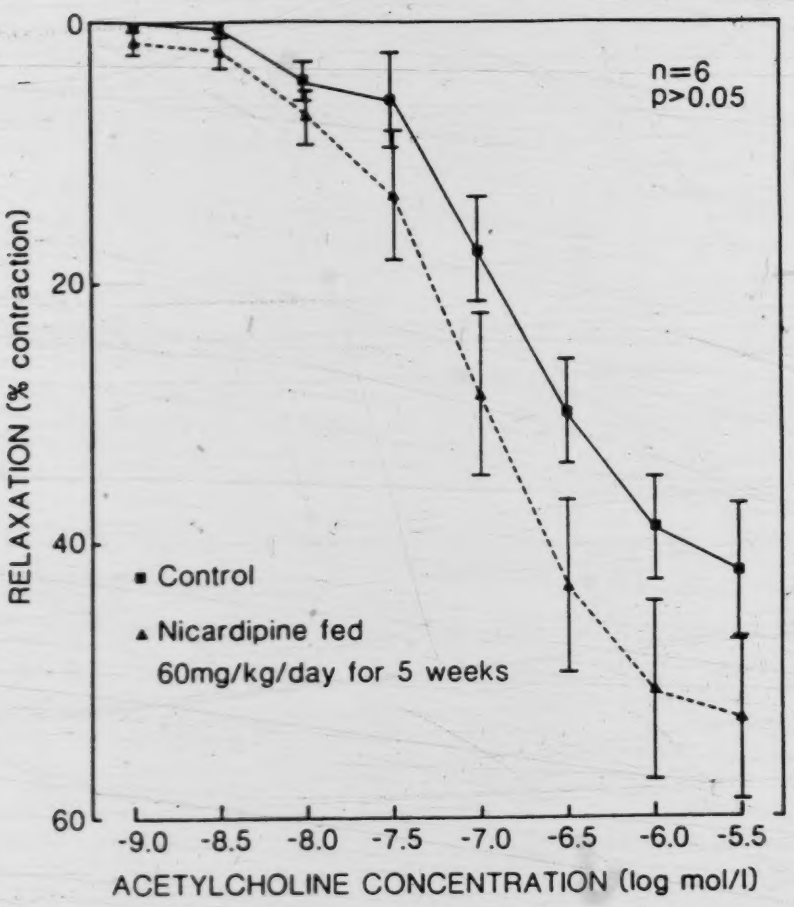

Figure 20. Cumulative concentration-effect curves to acetylcholine in control animals and animals fed nicardipine (60 mg/kg/day) for five weeks. The abscissa shows the concentration of acetylcholine and the ordinate shows the relaxation expressed as percent of the contraction to noradrenaline (-6.0 log mol/l). There was no significant inhibitory effect of nicardipine on endothelium-dependent relaxation to acetylcholine ($n = 6$, $p > 0.05$).

Table 12. The concentrations of nicardipine (μmol/l) in serum from rabbits fed nicardipine 60 mg/kg/day (as two divided doses) for 5 weeks (Rabbit Nos. CN1 - CN6). The last dose of nicardipine was given at 20:00 hours and the blood collected at 08:00 hours the next day at the time the rabbit was killed. No nicardipine was detected in the serum of control animals (Rabbit Nos. C2 and C4) which were not given nicardipine.

Rabbit No.	Serum nicardipine (μmol/l)
CN1	0.02
CN2	0.06
CN3	0.02
CN4	0.02
CN5	0.01
CN6	0.09
C2	0.00
C4	0.00

acetylcholine in the two groups of animals are shown in Figure 20 (n=6, p>0.05). No relaxation to acetylcholine was seen in the rings in which the endothelium was removed deliberately (maximum relaxations 0.0%, n=12).

The serum nicardipine concentrations in the two groups of animals are shown in Table 12. The detection of nicardipine in the serum of the animals orally fed nicardipine confirmed that the drug was absorbed into the circulation.

Protocol 4.2

The effect on EDR of incubating the rabbit aorta *in vitro* with nicardipine and diltiazem.

Tissue bath data for nicardipine

The aortic rings on exposure to noradrenaline (-6.0 log mol/l) showed a contractile response and the active tension generated was 7.6 ± 0.6 g (n=11). In the presence of nicardipine (-5.0 log mol/l), the active tension generated by the same concentration of noradrenaline was 7.3 ± 0.6 g. The responses observed without and with nicardipine were not significantly different (p >0.05). The relaxant response to acetylcholine was not significantly altered by nicardipine (Figure 21, p>0.05). The maximum relaxations without and with nicardipine were 32.4 ± 4.2% and 28.0 ± 3.1% of the contractile response to noradrenaline respectively (n=11, p >0.05).

Tissue bath data for diltiazem

Concentration-effect curves to acetylcholine were obtained before and after incubation with diltiazem (-5.0 log mol/l). The active tensions generated by noradrenaline before and after incubation with diltiazem were 5.7 ± 0.4 g and 7.6 ± 0.4 g respectively (n=11, p <0.05). The maximum relaxations to acetylcholine before and after diltiazem were 42.1 ± 5.7% and 36.4 ± 7.3% of the contractile response to noradrenaline (Figure 22). The difference between these results were not statistically significant (p >0.05).

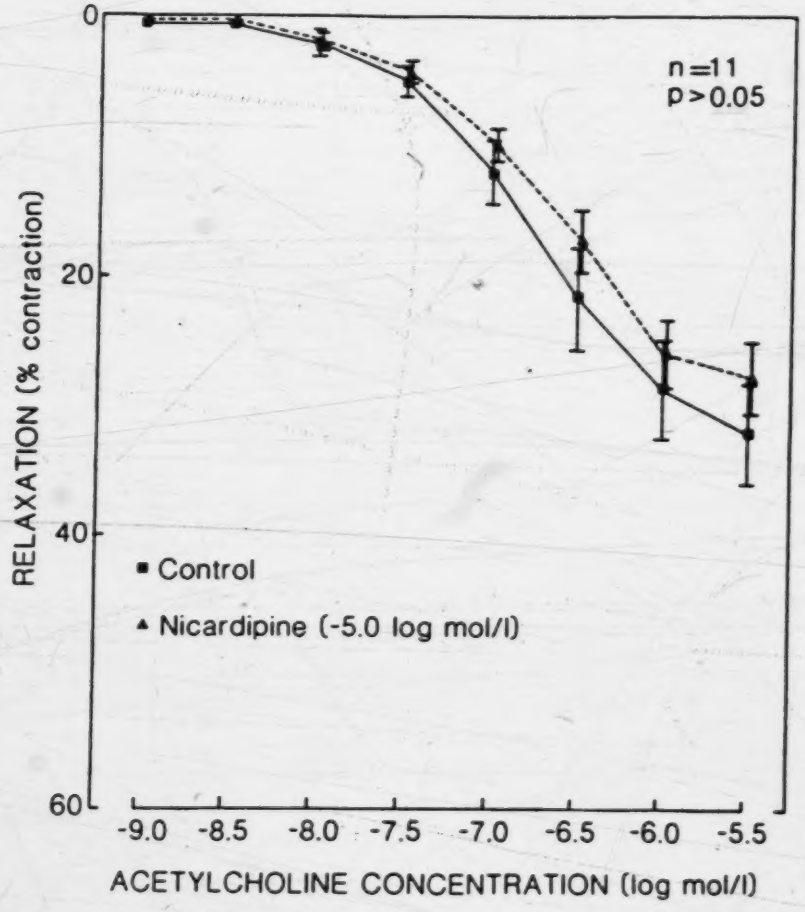

Figure 21. Cumulative concentration-effect curves to acetylcholine in control aortic rings and aortic rings exposed to nicardipine (-5.0 log mol/l) for 30 minutes. The abscissa shows the concentration of acetylcholine in log mol/l and the ordinate shows relaxation expressed as percent of the contraction to noradrenaline (-6.0 log mol/l). There was no significant difference between the two curves ($n = 11$, $p > 0.05$).

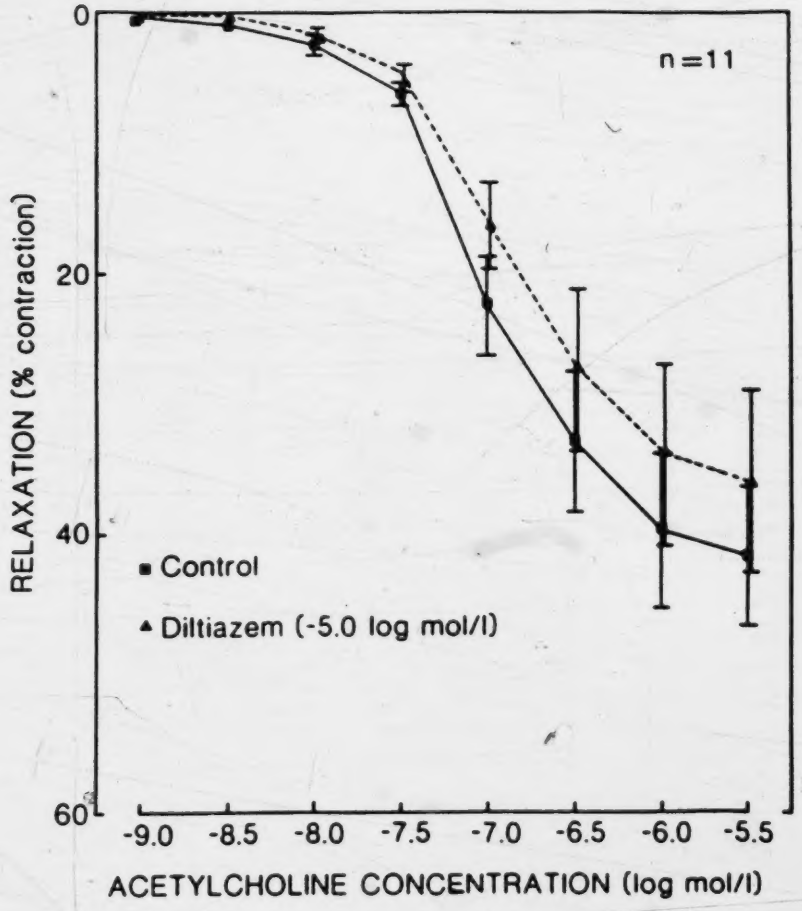

Figure 22. Cumulative concentration effect curves to acetylcholine in control aortic rings and aortic rings exposed to diltiazem (-5.0 log mol/l) for 30 minutes. The abscissa shows the concentration of acetylcholine in log mol/l and the ordinate shows the relaxation expressed as a percentage of the contraction to noradrenaline (-6.0 log mol/l). The results are not significantly different from each other ($n = 11$, $p > 0.05$).

Scanning electron microscopy showed that in the rings where the endothelium was not mechanically removed, over 80% of the endothelial surface was still intact at the end of the experiments. The endothelial surface appeared smooth with discernible cell margins.

Protocol 4.3

The effects of nicardipine on the synthesis/release of EDRF and on the effects of EDRF were assessed in this protocol.

Bioassay data

The protocol was completed in six preparations. Thus, six concentration-effect curves were generated in the control state, six curves were generated with the recipient alone exposed to nicardipine and six curves with nicardipine on both the donor and the recipient. Therefore, three sets of concentration-effect curves were compared in the final statistical analysis. The active tensions produced by prostaglandin $F_{2\alpha}$ in the three curves were 4.52 ± 0.43 g, 4.45 ± 0.42 g and 4.43 ± 0.44 g respectively. The maximum relaxations to acetylcholine were 23.5 ± 4.7%, 26.8 ± 5.0% and 27.2 ± 4.2% of the contractile response to prostaglandin $F_{2\alpha}$ respectively (Table 13). These results were not significantly different ($p > 0.05$). The relaxation responses to acetylcholine are shown in Figure 23.

Protocol 4.4

This protocol was included to confirm the calcium channel blocking properties of nicardipine and diltiazem under the present experimental conditions.

The aortic rings contracted on exposure to 100 mmol/l potassium. The active tension generated was 11.4 ± 1.3 g (n=6). In the presence of nicardipine (-5.0 log mol/l) the active tension generated by the same concentration of potassium was 6.4 ± 1.0 g. This tension was significantly different from that without nicardipine ($p < 0.05$). Similarly, the active tensions generated by potassium (100 mmol/l) before incubating with nicardipine (-4.0 log mol/l) and diltiazem (-4.0 log mol/l) were 10.0 ± 0.9 g and 14.5 ± 1.7 g

Table 13. Effects of nicardipine on recipient and on donor plus recipient. Relaxant responses to acetylcholine in recipient rings of rabbit aorta are expressed as a percent of the contraction to prostaglandin $F_{2\alpha}$.

	Concentration of acetylcholine (µmol/l)			
	0.5	1.6	5.3	16.6
Control(%)	1.4 ± 0.9	10.2 ± 2.6	13.1 ± 1.7	22.3 ± 5.2
Nicardipine on recipient (%)	1.7 ± 0.7	13.6 ± 3.5	14.8 ± 2.8	26.7 ± 5.5
Nicardipine on donor + recipient(%)	1.2 ± 0.6	7.8 ± 3.4	12.7 ± 2.7	27.2 ± 4.7

Responses are mean ± s.e. mean, n = 6. Prostaglandin $F_{2\alpha}$ at a concentration of 7.7 µmol/l was used. The control responses and the responses with nicardipine (83.8 µmol/l) on recipient and the responses with nicardipine (71.3 µmol/l) on donor (+ recipient) are shown.

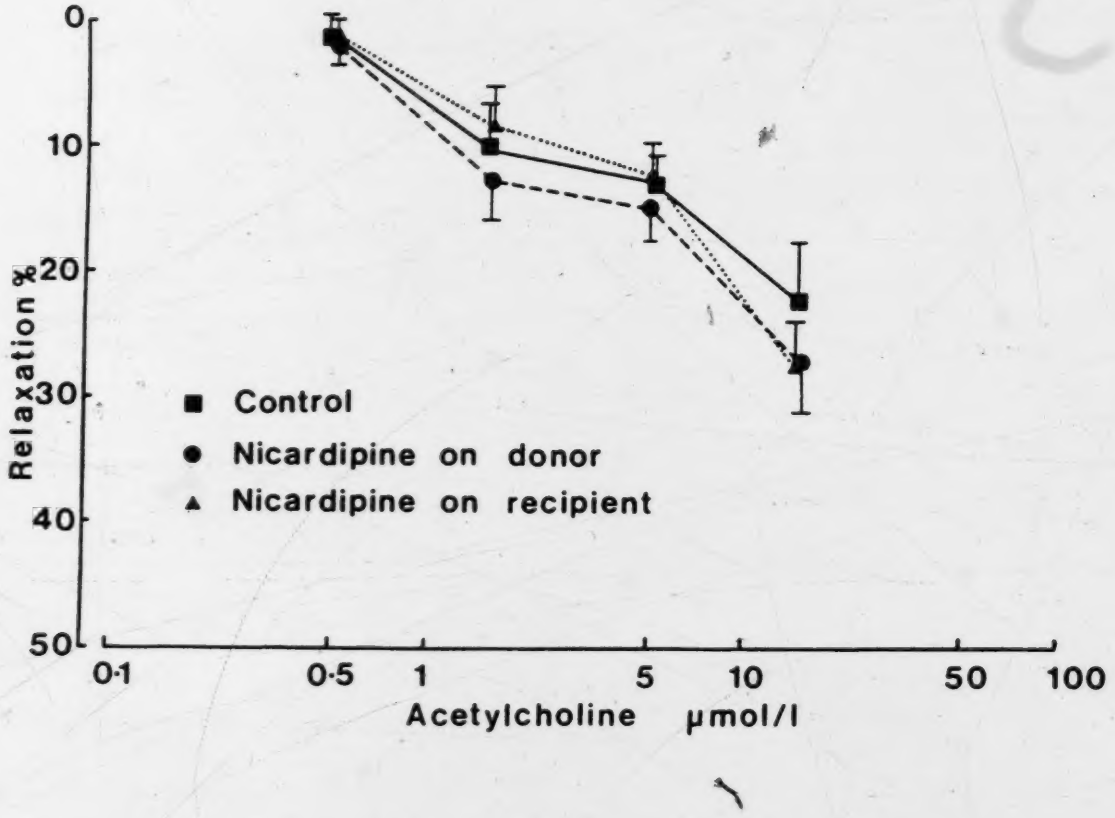

Figure 23. Responses obtained for acetylcholine from the bioassay protocol. The abscissa shows the concentration of acetylcholine (µmol/l) on a log scale and the ordinate shows the relaxation expressed as a percent of the contraction to prostaglandin $F_{2\alpha}$ (7.7 µmol/l). The responses to acetylcholine when nicardipine was infused on the recipient only, and on the donor and recipient were not significantly different from control ($n = 6$, $p > 0.05$).

respectively. The corresponding values after incubation with nicardipine and diltiazem were 3.3 ± 0.9 g and 7.8 ± 1.0 g (n=6, $p < 0.05$ for each).

SUMMARY

The EDR to acetylcholine in the aorta was not inhibited by feeding rabbits with the calcium channel blocker nicardipine at a dose of 60 mg/kg/day for 5 weeks. The EDR to acetylcholine was not influenced significantly by incubation with calcium channel blockers, nicardipine and diltiazem (-5.0 log mol/l) under *in vitro* conditions. The synthesis/release of EDRF and the relaxation effects of EDRF on vascular smooth muscle were not inhibited by nicardipine. Under the present experimental conditions, nicardipine and diltiazem inhibited the contractions elicited by 100 mmol/l potassium.

PROTOCOL FIVE

In this protocol, the EDR to acetylcholine was studied in the aortae of diabetic rabbits. The responses to noradrenaline and methoxamine were assessed also.

Protocol 5.1

A. Observations on animals and biochemical data

After injection of alloxan, within two days these animals showed glycosuria and elevated blood glucose concentrations. Over the next 14 days, they showed features of a complete diabetic syndrome including thirst, polyuria, weight loss, glycosuria, ketonuria and elevated blood glucose concentrations. Those animals who did not turn diabetic initially, when treated with a second injection of alloxan, showed features of diabetes mellitus. However, some animals were resistant even after two injections of alloxan.

Of the animals who developed diabetes, different grades of severity of the disease were seen. Some animals had fasting blood glucose concentrations of 450 mg%, while others had concentrations between 150-200 mg%. The weekly fluctuations of blood glucose concentrations in these animals are shown in Figure 24.

At the beginning of the study (before injecting alloxan), rabbits randomized to control and experimental groups weighed 2.6 ± 0.2 and 2.7 ± 0.1 kg respectively (mean ±

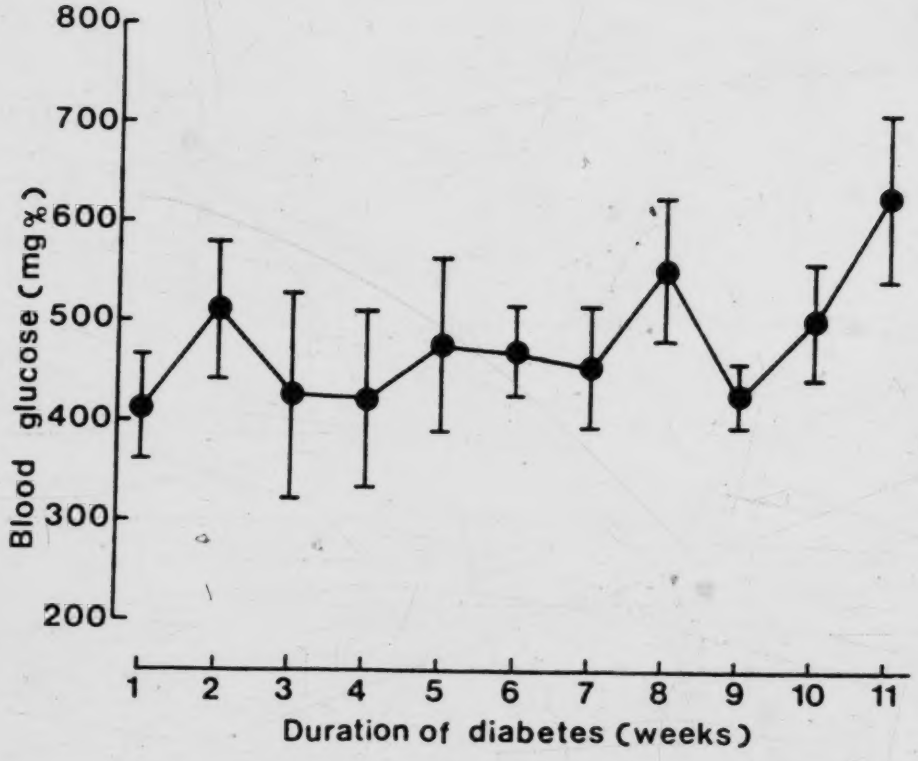

Figure 24. The blood glucose concentrations of diabetic rabbits. The abscissa shows the duration of diabetes in weeks and the ordinate shows the blood glucose concentration in mg% (mean ± s.e. mean, n = 6).

s.e. mean, n=6 for each). At the time of killing the control and diabetic rabbits weighed 4.1 ± 0.2 and 3.0 ± 0.3 kg respectively. In addition to the elevated blood glucose concentrations, another interesting feature seen in the diabetic rabbits was the markedly elevated serum triglycerides concentrations. These results are shown in Table 14. The diabetic rabbits also had slightly elevated cholesterol concentrations compared to controls. However, these concentrations were very much lower than those found in animals given the 2% cholesterol diet.

Except for the asthenic appearance of the diabetic rabbits no other differences were apparent externally. The external appearance of the abdominal organs were similar between the two groups of animals.

B. Appearance of aorta

The appearance of the aortae from diabetic rabbits were similar to the appearance from control rabbits. The intimal surface appeared smooth and no fatty spots or streaks were seen.

C. Sudan staining

Despite the elevated serum cholesterol and triglycerides concentrations, no sudanophilic areas were seen in the aortae of diabetic rabbits.

Electron Microscopy

The scanning and transmission electron microscopic appearances of the aortic endothelium in diabetic rabbits were similar to the appearances of control animals. These appearances had been described previously (protocol 1.1).

E. Tissue bath data

When examined in tissue baths, the aortic rings from control and diabetic rabbits had a mean basal tension of 6.5 ± 0.8 g and 5.8 ± 0.7 g, respectively (mean ± s.e. mean, n=6, $p > 0.05$). No spontaneous contractions were seen in these rings. When noradrenaline (-6.0 log mol/l) was added to the tissue bath, the aortic rings showed a contractile response. The active tensions generated in aortic rings from control and diabetic

Table 14. Data on control and diabetic rabbits.

	Control	Diabetic
Initial weight (kg)	2.6 ± 0.2 (n = 6)	2.7 ± 0.1 (n = 6)
At the time of killing		
Weight (kg)	4.1 ± 0.2	3.0 ± 0.3*
Blood glucose (mmol/l)	8.2 ± 0.7	27.2 ± 4.0*
Glycosuria	-	+
Ketonuria	-	+
Serum cholesterol (mg%)	47.9 ± 3.9	134.8 ± 24.3*
Serum triglycerides (g/l)	1.86 ± 0.40	22.20 ± 6.00 *
Aortic rings		
Basal tension (g)	6.5 ± 0.8	5.8 ± 0.7
Active tension (g)	5.4 ± 0.8	5.3 ± 0.4

* $p > 0.05$, Active tension refers to the tension generated by noradrenaline (-6.0 log mol/l).

(- = absent; + = present)

animals were 5.4 ± 0.8 g and 5.3 ± 0.4 g respectively ($p > 0.05$). With the addition of acetylcholine, a concentration dependent relaxation was seen. At concentrations higher than -5.0 log mol/l acetylcholine, the relaxation response reversed and a contractile response was seen. The maximum relaxations to acetylcholine in aortic rings from control and diabetic animals were 73.1 ± 7.9% and 74.8 ± 7.6% of the contraction to noradrenaline respectively. The concentration-effect curves for acetylcholine in the aortae of the two animal groups are shown in Figure 25. These curves were not significantly different from each other ($p > 0.05$).

Effect of sodium nitrite

No significant differences were seen in the relaxant responses to sodium nitrite (-4.0 and -3.0 log mol/l) in aortic rings from control and diabetic animals ($n = 6$, $p > 0.05$). The maximum relaxations elicited by sodium nitrite (-3.0 log mol/l) in aortae from control and diabetic rabbits were 74.7 ± 10.4% and 73.9 ± 10.0% of the contraction to noradrenaline, respectively.

Responses to the α adrenoceptor agonists

The basal tension in aortic rings from control and diabetic animals were 5.8 ± 1.3 g and 6.2 ± 1.0 g respectively (mean ± s.e. mean, $n = 5$). With the addition of noradrenaline, the aortic rings produced a contractile response which reached a maximum at a noradrenaline concentration of -4.0 log mol/l. The maximum tension generated by aortic rings from control and diabetic animals were 9.4 ± 0.4 g and 11.2 ± 2.0 g respectively ($p > 0.05$). The slopes and ED_{50} values obtained from concentration-effect curves of the percentage responses of control and diabetic animals were not significantly different from each other (ED50 values: control, 0.55 μmol/l; diabetic, 0.65 μmol/l).

Methoxamine produced a concentration dependent contraction in the aortae of diabetic rabbits which was not significantly different from the control ($p > 0.05$). The active tensions generated by methoxamine in aortic rings from control and diabetic animals were 7.4 ± 1.4 g and 9.8 ± 1.9 g respectively. The slopes and ED_{50} values of the

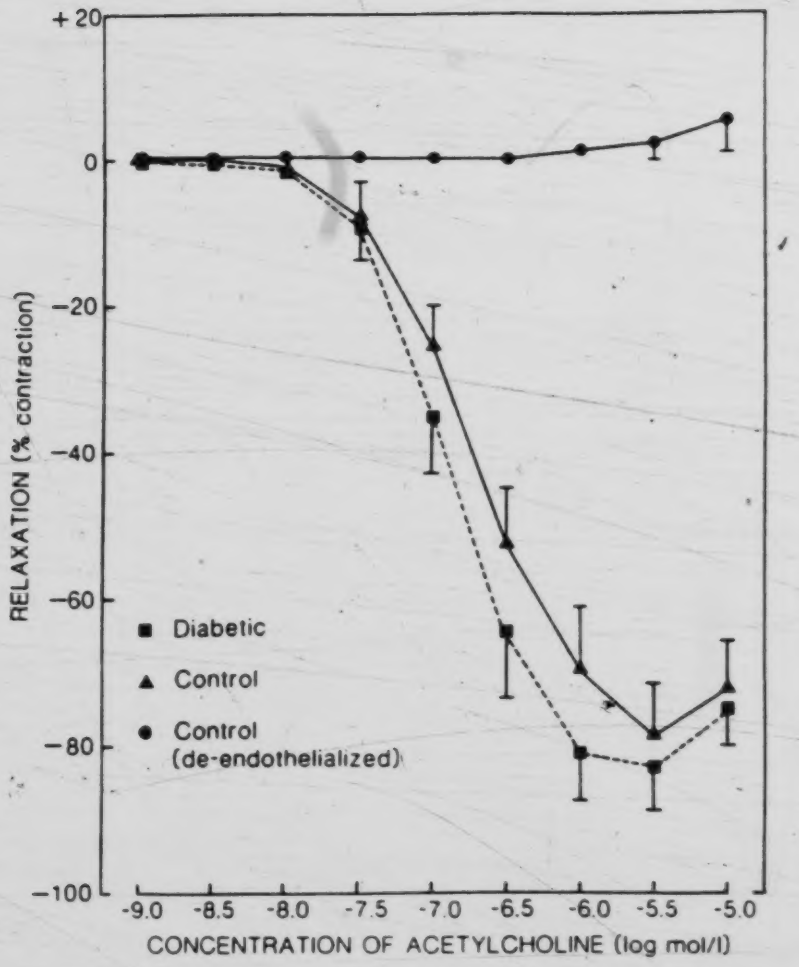

Figure 25. Cumulative concentration-effect curves to acetylcholine during contractions to noradrenaline in control and diabetic rabbits. The abscissa shows the log mol/l concentration of acetylcholine and the ordinate shows the relaxation expressed as percent of the contraction to noradrenaline (-6.0 log mol/l). The responses between control and diabetic animals were not significantly different ($n = 6$, $p > 0.05$). The rings without endothelium showed a contractile response at higher concentrations of acetylcholine. -: relaxation, +: contraction.

concentration-effect curves between control and diabetic animals were not significantly different from each other ($p > 0.05$). The ED_{50} values for control and diabetic animals were 2.71 and 2.37 µmol/l respectively.

Contractile responses elicited by the selective α_1 adrenoceptor agonist methoxamine and the non-selective α adrenoceptor agonist noradrenaline were not significantly different between control and diabetic animals ($p > 0.05$).

SUMMARY

In the rabbit model of experimental diabetes mellitus, the EDR to acetylcholine was not impaired in the aorta.

SUMMARY

The results described in the preceding protocols can be summarized as follows.

Effect of 2% cholesterol feeding on EDR to acetylcholine

Atherosclerosis was induced in rabbits by feeding a diet containing 2% cholesterol and lipids. The vascular lesions produced by atherosclerosis were studied by sudan staining, light and electron microscopy. Fatty spots, streaks and plaques were seen in the aortae of animals given this diet. These abnormalities were not seen in aortae of control rabbits given standard rabbit diet.

When studied at 4, 8 and 10 weeks while on the 2% cholesterol diet the EDR to acetylcholine was impaired in the aortae of these animals.

In some rabbits, the food was reversed to standard rabbit diet after an initial 6 weeks of 2% cholesterol feeding and studied after 14 and 32 weeks (reversal study). The EDR to acetylcholine was persistently impaired in the aortae of these rabbits.

Bioassay experiments

A bioassay technique for measurement of activity of EDRF was developed. The bioassay experiments suggested that impairment of EDR in cholesterol-fed rabbits was due to an impairment in synthesis/release of EDRF. This impaired synthesis/release of EDRF persisted for 36 weeks after reversal of diets.

Effect of age and 2% cholesterol diet on EDR to acetylcholine

Young (8 weeks of age) and older (46 weeks of age) rabbits were given the 2% cholesterol diet for 4 weeks and the EDR to acetylcholine was assessed. The EDR to acetylcholine was impaired in the aortae of both young and older rabbits. In the young rabbits the impairment of EDR was associated with intimal and subintimal lesions seen in classical atherosclerosis. In the older animals, the impairment of EDR was not associated with obvious intimal and subintimal lesions seen with atherosclerosis. Only microscopic evidence of endothelial cell damage was seen.

Effect of calcium channel blockers on EDR to acetylcholine

In the rabbit aorta, the calcium channel blockers nicardipine and diltiazem were found to have no significant inhibitory effect on EDR to acetylcholine.

Diabetes and EDR

Unlike in experimental atherosclerosis, the EDR to acetylcholine was not impaired in the aortae of rabbits made diabetic by injecting alloxan.

DISCUSSION

The present investigation has demonstrated that pre-contracted aortic rings taken from control and atherosclerotic rabbits, when studied in tissue baths showed a relaxant response to acetylcholine. This relaxation was found to be maximal at acetylcholine concentrations of -6.0 to -5.5 log mol/l. The concentration-effect curves for acetylcholine were shifted to the right by atropine (-8.0 log mol/l). The cyclooxygenase inhibitor indomethacin (-6.0 log mol/l) had no significant inhibitory effect on this relaxation. The lipooxygenase inhibitor NDGA (-4.4 log mol/l) antagonized the relaxation. Also, the EDR was lost on removal of the endothelium. All these features suggest that the relaxant response observed in the aortae in the present study is consistent with the EDR described by Furchgott (1983).

Impairment of EDR to acetylcholine in cholesterol-fed rabbits

The experiments described in Protocol One of this study were designed to test the hypothesis that atherosclerosis induced by feeding a high cholesterol diet may be accompanied by an impairment of EDR. The results of this protocol showed that there was a persistent impairment of EDR to acetylcholine in the aortae of rabbits fed the high cholesterol diet. Similar results have been published by Chappell *et al.*, (1987). This impairment could be due to one or more of several causes:

1. Destruction of isolated endothelial cells.
2 Impaired production of EDRF.
3. Impaired diffusion and enhanced degradation of EDRF during transit from endothelium to underlying smooth muscle.
4. Impaired relaxation of aortic smooth muscle to EDRF.

1. Destruction of isolated endothelial cells.

An intact functioning endothelium has been shown to be essential for eliciting EDR to acetylcholine in the rabbit aorta (Furchgott and Zawadzki, 1980a). However, Peach *et al.* (1985) have concluded that in large arteries 30 - 35% of endothelial cell loss is tolerated

without impairment in EDR. In smaller arteries, this value may go up to about 65 - 75%. In the present study, scanning electron microscopy of the atherosclerotic aortic preparations where the EDR to acetylcholine was found to be impaired, showed that over 80% of the endothelium was intact. Hence loss of isolated endothelial cells is unlikely to be the cause for the impairment of EDR seen in this study.

2. Impaired production of EDRF.

In the present study, several abnormalities in the endothelial cells were revealed by scanning and transmission electron microscopy. These anomalies were of a diffuse nature. The endothelial cells showed accumulation of lipids in their cytoplasm and appeared oedematous. In some cells, the nuclei showed features suggestive of degeneration. Large accumulations of intra-and extracellular lipids were seen in the subendothelial tissues. Lipid laden foam cells were seen also. These features are similar to those described by previous investigators (Imai *et al.*, 1966; Parker and Odland, 1966). These abnormalities in the endothelial cells could influence the synthetic machinery in the cells leading to an impaired production of EDRF and hence to an impaired relaxation to acetylcholine.

3. Impaired diffusion and enhanced degradation of EDRF during transit.

As described previously, numerous lipid deposits were seen in the subintimal tissues of the atherosclerotic arteries. Under the light microscope, several layers of lipid laden cells were seen between the endothelium and vascular smooth muscle. These changes caused an actual thickening of the subintimal tissues. Such lipid deposits could act as a diffusion barrier and delay the EDRF from reaching the vascular smooth muscle. As EDRF has been shown to be an unstable metabolite with a half life of about 6 seconds, it is likely to undergo degradation during transit (Griffith *et al.*, 1984). Hence, in atherosclerotic aortae with structurally altered subintima, impaired diffusion and enhanced degradation of EDRF can occur. This will manifest as impaired EDR to acetylcholine as seen in the present study.

4. Impaired relaxation of vascular smooth muscle.

Although changes due to atherosclerosis were prominent in the subintimal tissues, changes were seen in the tunica media of the atherosclerotic arteries also. Deposits of lipids were seen in the sarcoplasm and between muscle cells. Foci of degeneration of fibrous tissue were seen also. Such changes could influence smooth muscle function leading to impaired relaxation to EDRF.

Hence, except for the destruction of isolated endothelial cells, all the other three causes discussed above could have accounted for the impairment of EDR to acetylcholine seen in the present study. The bioassay protocol was designed to resolve these issues.

In the bioassay experiments (Protocol 2.2), the EDRF generated from paired donor aortic preparations obtained from control and atherosclerotic animals were assayed on a single common recipient. The deendothelialized recipient tissue was obtained from a control rabbit. The principal finding in this protocol was that the relaxant responses produced in the recipient by acetylcholine acting on atherosclerotic donors were significantly impaired compared to those produced by control donors.

As the flow rates of the pumps were kept constant and equal sized donor aortae were used, it is assumed that each donor aorta is stimulated in an equal fashion to release similar amounts of EDRF. On release of EDRF it is carried in the perfusate to act on the recipient. As tubings of equal length and diameter were used between the two donors and the recipient, whatever inactivation of EDRF that occurred should be similar in both limbs of the bioassay system. Hence, the differences in EDR elicited in the recipient is likely to be due to differences in the amount of EDRF released from the two donors.

The relaxant responses obtained in the recipient for each concentration of acetylcholine tested on atherosclerotic donors were less compared to those tested on control donors (maximum relaxation $16.5 \pm 4.9\%$ for atherosclerotic donors; $32.7 \pm 5.3\%$ for control donors, $n = 10$, $p < 0.05$). This finding suggested that, the production of EDRF in atherosclerotic aortae may be about 50% less compared to control aortae. When the experiment was repeated with the recipient assay tissue from an atherosclerotic animal,

a similar pattern of results (i.e., the relaxation obtained by the atherosclerotic donor aorta was impaired compared to the relaxation obtained by the control donor aorta) was seen. These results confirmed that the production of EDRF is impaired in the aortae showing atherosclerosis.

Verbeuren *et al.* (1986) using the rabbit aorta in a bioassay system failed to demonstrate any differences in the production of EDRF between control and atherosclerotic arteries. However in their study, instead of the thoracic aorta, the abdominal aorta was used. The difference in results reported by Verbeuren *et al.*, compared with results of this study may be due to differences in the extent of atherosclerosis seen between the thoracic aorta and the abdominal aorta. In the rabbit model of experimental atherosclerosis the thoracic aorta is diseased to a greater extent compared to the abdominal aorta. In a subsequent study, using the thoracic aorta as the donor tissue of EDRF, the same authors showed that production of EDRF is impaired in atherosclerosis (Verbeuren *et al.*, 1987).

Bossaller *et al.* (1987) in a study using atherosclerotic rabbit aorta and atherosclerotic human coronary arteries showed that EDR to acetylcholine was impaired in these vessels. However, they also found that the EDR to the calcium ionophore A23187 was not impaired in the same vessels. On this evidence, they concluded that there is a selective cholinergic impairment in atherosclerotic blood vessels. However, others have shown that EDR to both acetylcholine and A23187 are impaired in atherosclerotic rabbit aortae (Goodwin *et al.*, 1987). The ionophore is thought to by-pass the receptor and stimulate release of EDRF. Hence, the latter experiments suggest that the defect leading to impairment of EDR is distal to the level of the muscarinic receptors.

One of the reasons discussed as a cause of impairment in EDR to acetylcholine in atherosclerosis was impaired diffusion and increased degradation of EDRF. However, evidence from a recent study by Cocks *et al.* (1987) argues against impairment of diffusion of EDRF as a cause for impairment of EDR. In their study, the release of EDRF was examined in the rabbit carotid artery 6 weeks after denudation with a balloon catheter *in*

vivo. A concentric fibromuscular intimal thickening of variable thickness developed in all areas lined with regenerated endothelium. These reendothelialized areas with large concentric thickening (between 20 - 30 cells thick) relaxed with a similar sensitivity and maximum to methacholine compared with control areas. Hence, it was shown that the presence of a large fibromuscular intima did not prevent EDRF from reaching the media to cause relaxation. However, these authors stated that in models of hypercholesterolemia, the barrier produced by the large lipid deposits could be different compared to the large fibromuscular intima in the above situation. Such lipid deposits may function as physical barriers for diffusion of EDRF from endothelium to smooth muscle.

The data from Protocol 2.2 of the present study also argue against an impaired diffusion of EDRF from endothelium to the underlying smooth muscle as a cause for impairment of EDR to acetylcholine. As part of this protocol the relaxant responses elicited by a common control donor in two different recipient tissues (one from control and the other from atherosclerotic animals) were compared. In this experiment no difference in the degree of relaxation was seen between the two recipients. If impaired diffusion of EDRF were an important cause for impaired relaxation to acetylcholine, then, the relaxations in the recipient tissues obtained from the atherosclerotic animals should have been impaired. As no such impairment in relaxation was noted, impaired diffusion and increased degradation of EDRF does not appear to be an important cause leading to impaired EDR.

In the present study the function of the vascular smooth muscle of atherosclerotic arteries was studied also. The contractile function was assessed as concentration-effect curves to the α adrenoceptor agonists. No significant differences in sensitivity to noradrenaline, methoxamine and clonidine (protocol 1.3) were seen between the aortae of control and atherosclerotic rabbits. Hence, the contractile function of the vascular smooth muscle did not appear to be influenced by atherosclerosis. Similarly, no difference was seen in the relaxation response to the endothelium-independent vasodilator, sodium nitrite. This finding suggests that relaxation of vascular smooth muscle *per se* is not impaired in

the atherosclerotic rabbit aorta. In the study by Bossaller *et al.* (1987), no differences were seen in the amounts of relaxation to sodium nitroprusside between control and atherosclerotic arteries. In their study the amount of cGMP generated by control and atherosclerotic arteries were also found to be not different. These findings are in agreement with those of the present study.

Findings different from above have been reported by Verbeuren *et al.* (1986). In their study, impaired relaxations to both acetylcholine and nitroglycerin were reported in the arch of the aorta of atherosclerotic rabbits. As the relaxation to the endothelium-independent vasodilator, nitroglycerin, was impaired also, these authors concluded that smooth muscle relaxation is impaired in the more severely affected aortic arch. It must be mentioned that the findings pertaining to the aortic arch may not be representative of the smooth muscle function of the rest of the aorta, especially because the same authors found no impairment in relaxation to nitroglycerin in the abdominal aorta.

The experiments addressing this issue of smooth muscle function of atherosclerotic arteries have been conducted without making appropriate corrections for physical properties of tissues like length and weight. Hence, proper comparisons may not be possible between control and atherosclerotic vessels. Further studies will be needed to resolve this particular issue.

The results discussed in this section confirm the hypothesis that impairment of EDR in experimental atherosclerosis is due to impaired production of EDRF. Impaired diffusion of EDRF has been shown not to be an important reason for impairment in EDR to acetylcholine in atherosclerotic arteries.

Before proceeding further with the discussion, a critique of the bioassay technique used in the present study seems appropriate. In this technique, the following measures were taken to minimize variation in the experiments enabling valid comparisons to be made.

1. The flow rates of the pumps were kept constant throughout the experiment. This enabled equal amount of intra-luminal perfusate to be delivered onto the recipient.

2. The donor aortae from control and atherosclerotic animals were cut to equal lengths from corresponding areas of the descending thoracic aorta.
3. Fixed concentrations of drugs (noradrenaline, acetylcholine and atropine) were used throughout the study.
4. The initial preload on the recipient was kept constant.
5. When two recipients were used, the initial length at which the isometric preparation was set and the degree of induced contraction were kept within comparable limits.
6. Recipient tissues of equal length were used.

Limitations of the technique

1. A major limitation of the technique is that a reproducible dose-response relationship has not been demonstrated. In these experiments, responses to only four concentrations of acetylcholine were assessed. Usage of concentrations to cover both lower and higher ranges of the concentration-effect relation for acetylcholine would have been more useful. However, as continuous stimulation of the endothelium to release EDRF for several hours could lead to anomalies in release of this substance, the protocol was kept short.
2. In tissue bath experiments, the tissue is incubated in a bath of fixed volume and drugs are added to the tissue bath. As rapid equilibration occurs due to O_2-CO_2 bubbling, the exact concentration of the drug can be deduced. However, in the bioassay technique, drugs are injected into the perfusate and it is assumed that complete mixing and equilibration occurs inside the lumen of the donor, in the connecting tubes and on the recipient tissue. However these assumptions may not be met on every occasion.

In Protocol Three, the hypothesis that impairment of EDR to acetylcholine is an early marker of atherosclerosis was tested. In this protocol, the effect of the atherogenic diet on rabbits of two age groups [8 weeks of age (young) and 46 weeks of age (older)] was assessed. The appearances of the aortae of the young rabbits given the atherogenic

diet were typical of experimental cholesterol atherosclerosis. The EDR to acetylcholine was found to be impaired in these aortae. When the older rabbits were given the atherogenic diet for 4 weeks, the results appeared to differ from those in the young rabbits. The consumption of food by the older rabbits was less compared to the young rabbits resulting in a lesser degree of hypercholesterolemia. Fatty spots, streaks and plaques were not seen in the aorta. The sudan grading of these specimens was 0.2 with only one specimen out of nine demonstrating positive sudan staining. Under the light microscope, no sub-endothelial lipid deposits were seen in these animals. In fact, the light microscopic appearances were similar to those of control animals given a standard rabbit diet. However, scanning electron microscopy revealed an abnormal endothelial surface. The appearances were similar to those described on the endothelium of young rabbits fed a high cholesterol diet (Rodman *et al.*, 1979). When viewed under the transmission electron microscope, the endothelial cells showed vacuolation of cytoplasm. The several layers of lipid laden cells seen in the subendothelial areas of the young rabbits given the atherogenic diet were not seen in these older rabbits. Hence, the ultrastructural abnormalities were mainly restricted to the endothelium of the older animals. When the EDR to acetylcholine was assessed this was found to be impaired in the aorta.

Thus, in this protocol, in the young rabbits the impairment of EDR was associated with the occurrence of changes of classical atherosclerosis in the aorta. However, in the older rabbits, the impairment of EDR was dissociated from the occurrence of changes of classical atherosclerosis in the aorta. It may be said, that in young rabbits, impairment of EDR was associated with both hypercholesterolemia and atherosclerosis while, in older rabbits, the impairment of EDR was associated with hypercholesterolemia without atherosclerosis.

In the present study, the aortic endothelium of the older rabbits appeared structurally abnormal. The impairment of EDR in these aortic rings suggests an impairment in the synthesis/release of EDRF from the endothelial cells. An impairment in diffusion

and/or an increased degradation of the relaxation factor during transit from the endothelial cells to the smooth muscle appears less likely in view of the paucity of demonstrable changes in the subendothelial layer. Hence, impairment of EDR may occur at an early stage in the process of atherosclerosis. These results support the hypothesis that impairment of EDR is an early marker of atherosclerosis. However, as the older animals had significant loss of weight, it is possible that impairment of EDR is completely independent of atherosclerosis also.

Mitchell *et al.* (1986) postulated that, since veins exposed to hypercholesterolemia do not develop atherosclerosis, the effect of hyperclolesterolemia *per se* on EDR could be best assessed in venous preparations. Hence, the responses to acetylcholine in jugular veins from monkeys with arterial atherosclerosis (induced by feeding an atherogenic diet for 18 months) and normal monkeys were studied. No significant difference was seen in the EDR to acetylcholine between these two groups of vessels. In a similar study using normolipidemic and hyperlipidemic dogs, the same authors obtained similar results. They concluded that impaired EDR in atherosclerosis was not due to hypercholesterolemia alone, but required the presenoe of atherosclerosis. They also postulated that the abnormality of EDR in atherosclerotic arteries may be due to an intimal barrier or to endothelial damage related to atherosclerosis. These results in the monkey and dog models appear to be different from the findings in the older rabbits. However, in the study by Mitchell *et al.*, no information about the endothelial cell morphology was provided.

The effect of age on susceptibility of rabbits to experimental atherosclerosis is not clear. In a study feeding 1% cholesterol and 5% vegetable oil to rabbits, Harman (1962) concluded that young, rapidly growing rabbits developed more aortic atherosclerosis on an *ad libitum* diet compared to older, more slowly growing rabbits. He postulated that there was a greater affinity of newly formed tissue to lipids. In another study on rabbits, using 0.1 g of cholesterol per 600 g body weight given over 30, 45 and 60 days, Pollak (1947) concluded that older rabbits were more susceptible to atherosclerosis.

In the present study (Protocol Three), a greater susceptibility of young rabbits for developing hypercholesterolemia and atherosclerosis (when fed a diet supplemented with cholesterol) compared to older rabbits was noted. This effect has been shown previously also (West *et al*, 1982). The findings in the present study are complicated by the fact that older rabbits demonstrated a significant loss of weight during the period of high cholesterol feeding. This effect could be attributed to a reduced intake of food (and cholesterol) during the period of study leading to a lower serum cholesterol concentration and a lesser degree of atherosclerosis. In addition, it has been reported that older rabbits have a reduced absorption of cholesterol (Thomson, 1981). The effect of loss of weight on the morphological changes observed in the endothelium remains unclear at present. Hypercholesterolemia has been shown to occur with weight loss in rabbits (Swaner and Connor, 1975). Thus, the loss of weight observed in the present study may have contributed to the hypercholesterolemia which in turn contributed to the loss of EDR demonstrated in this study.

The results discussed above favour the hypothesis that the susceptibility to atherosclerosis varies as a function of the age of the animal and possibly the plasma cholesterol levels. The young rabbits had a greater susceptibility to atherosclerosis compared to older rabbits. However, the results pertaining to the older rabbits were confounded by the loss of weight in these animals.

Non-regression of atheroma and persistent impairment of EDR

In the preceding sections, it was shown that feeding an atherogenic diet to New Zealand White rabbits led to hypercholesterolemia and eventually, to a form of atherosclerosis. In this model, the EDR to acetylcholine was found to be impaired in the aorta. This impairment was shown to be due to an impaired production of EDRF.

In Protocols 1-2 and 2-3, after an initial six weeks of cholesterol feeding, the diet was reversed to standard rabbit food and the animals followed up for a further 14, 32 and 36 weeks. Two questions were addressed in these 'reversal' experiments.

1. Is there a recovery in the production of EDRF in reversal animals?
2. Is there regression of atherosclerosis in the rabbit aorta?

1. Is there a recovery in the production of EDRF in reversal animals?

When studied in tissue baths, the EDR to acetylcholine was found to be impaired after 32 weeks following the reversal of diets. Further, the bioassay method demonstrated that the impairment of EDR was due to decreased production of EDRF. This persistent functional impairment may be due to the following reasons:

(i) slow turnover of endothelial cells

(ii) regeneration with diseased cells and

(iii) continuing damage to regenerated normal endothelium.

(i) Slow turnover of endothelial cells

The rate of turnover of endothelial cells has been determined using the tritiated thymidine technique. It is generally believed that the rate of cell turnover of vascular endothelium is slow (Schwartz, 1983). In young rabbits (6-10 weeks of age), the life span of an endothelial cell is believed to be about 7 - 8 weeks while in adult rabbits (16-48 weeks of age), it is believed to be about 24 weeks (Kunz, 1983). The incorporation of thymidine in older animals is a measure of cell renewal (replacement of dead cells) while in the young animals, it represents a proliferative process (due to growth of tissue) as well as cell renewal (Kunz, 1983).

Although the overall cell turnover rate in the normal endothelium is slow, foci of high spontaneous cell turnover has been described (Schwartz *et al.*, 1980). In addition, the cell turnover has been shown to be increased in conditions of hyperlipidemia (Florentin *et al.*, 1969; Hassler, 1971). When animals initially fed a high cholesterol diet are put back on standard diet, a mild hypercholesterolemia is reported to persist for some time (Albrecht and Schuler, 1965). However, with time, the cholesterol concentrations return to those of control animals. In the present study, the serum cholesterol concentrations in the reversal animals declined to values seen in control animals after 12 weeks of reversal of diets.

Under the experimental conditions of this study, the prolonged hypercholesterolemia would act as a stimulus for increased cell turnover in the reversal animals. In addition, a sufficient period of time (36 weeks) was allowed to lapse after reversal of diets. Hence, under these conditions the cell turnover rate will not be slow, if at all, it will be fast .

Therefore, a slow turnover of endothelial cells is unlikely to be the mechanism behind the persistent impairment of EDR in the reversal animals.

(ii) Regeneration with diseased cells

Autoradiography of the endothelium has shown the presence of replicating cells (Florentin *et al*, 1969). Studies from several laboratories have suggested that areas of high endothelial turnover may occur at sites in the arterial tree that are prone to the development of atherosclerosis (Wright, 1972; Caplan and Schwartz, 1973). Under the present experimental conditions, it is possible that the increased level of serum cholesterol might have caused permanent damage to the nuclei of replicating cells leading to the production of abnormal new cells. Such abnormal new cells may be deficient in their ability to produce EDRF. Thus, regeneration with diseased endothelial cells may be a mechanism for the persistent impairment of production of EDRF seen in the reversal animals.

(iii) Continuing damage to regenerated normal endothelium

It is possible that following reversal of diets, the endothelium underwent regeneration and new cells were produced. These regenerated normal endothelial cells might have allowed continued entry of lipids into the regenerated endothelium and to the underlying intima. Such a state could lead to continued damage to the endothelial cells. This proposition has been investigated by studying the regeneration of the endothelium after balloon catheter injury. Such studies have shown that, in rabbits, lipid accumulates preferentially in areas covered by regenerated endothelium (Minick *et al*., 1977; Rosenfeld *et al*., 1983). This accumulation of lipids appears to be due to metabolic differences in the reendothelialized areas. Such differences are thought to persist for up to one year after a single balloon catheter de-endothelialization and re-endothelialization cycle. This continued

entry of lipids will damage the regenerated endothelium. Such a state could lead to continued impairment of production of EDRF.

In the preceding discussion three factors were considered as possible causes for the persistent impairment of production of EDRF in the reversal animals. Of these three factors, slow turnover of endothelial cells does not appear to be an important contributory factor. The data presented in this study does not help to separate which of the other two factors (i.e., regeneration with diseased cells and continuing damage to regenerated normal endothelium) are responsible for the impaired production of EDRF in the reversal animals. However, considering the findings of the "balloon catheter - injury model" experiments, it is likely that there is continuing damage to the regenerated endothelium leading to impaired production of EDRF.

2. Is there regression of atherosclerosis in the rabbit aorta?

As mentioned previously, in the reversal animals, the serum cholesterol and triglycerides concentrations returned to values found in control rabbits about 12 weeks after reversal of diets. Hence, as the endothelium is no longer exposed to the high concentrations of lipids, one could expect the atherosclerotic changes in the intima and media to either regress or at least not progress further. However, no regression of atherosclerosis was seen in these animals. In fact, a further accumulation of cholesterol and lipids was seen in the aorta after reversal of diets. This was confirmed by the increased aortic tissue cholesterol concentration and the worsening of the grading of sudanophilia in these animals. These findings are consistent with the observations of McMillan *et al.*, (1955).

When studied under the scanning electron microscope, some features of "clearing" of the abnormalities of the intimal surface were seen in the aortae of reversal animals. The endothelium formed a continuous lining in most areas and the cell borders were becoming distinct. These features are similar to those described by Weber *et al.* (1975). However, under the light microscope, the intimal surface was still covered with plaques. Under the

transmission electron microscope, persistent structural abnormalities were seen in the endothelium. Vacuolation of endothelial cells and foam cells in the subendothelial areas were still seen. It could be postulated that the abnormal endothelium allowed the entry of lipids from the luminal surface into the intima and media. Thus, although the serum cholesterol concentration returned to control values, continued accumulation of lipids occurred. Hence, under the present experimental conditions, no regression of atherosclerosis was seen in the rabbit model. Similar results have been observed by Adams *et al.*, (1973). These results support the hypothesis that abnormalities in the endothelium may allow continued accumulation of lipids in the sub-endothelial cell layers. There is a clear clinical implication if this hypothesis is correct: the prevention of endothelial cell abnormalities would be of paramount importance since the correction of hypercholesterolemia after the initiation of endothelial cell injury may be insufficient to prevent the progression of the atherosclerotic lesions.

In the studies discussed here, the 2% cholesterol diet was given to the animals for 6 weeks. It would be interesting to investigate whether reversal could be demonstrated with a shorter initial exposure to the high cholesterol diet.

These observations are in contrast with those reported by Armstrong *et al.*, (1970) in the monkey. In the monkey, reversal of a diet containing high concentrations of cholesterol was accompanied by regression of atherosclerotic lesions (Armstrong and Megan, 1972). This process of regression was accompanied by a reduction in the thickness of the tunica intima with fibrosis within the intima (Armstrong *et al.*, 1970). Recent studies from the same group demonstrated a restoration of EDR to acetylcholine in iliac arteries taken from monkeys fed a high lipid (0.7% cholesterol, 40% lipids) diet for 18 months followed by standard monkey chow for a further 18 - 20 months (Freiman *et al.*, 1986). Further, the relaxant response to thrombin was restored to normal in these animals. Although the findings in the monkey differed from those observed in the rabbit it is not

known which of these two animal models of atherosclerosis more closely mimics the human condition.

Diabetes Mellitus and EDR

In humans, diabetes mellitus is a risk factor associated with increased incidence of atherosclerosis and myocardial infarction (Ross, 1986).

The mechanisms as to how diabetes mediates its effects remain poorly understood. The effects of diabetes may be mediated through abnormal lipoproteins that are usually seen in uncontrolled diabetes. Alternatively, the influence of diabetes may be independent of the lipid profile in the blood. In Protocol Five, the hypothesis that a diabetic state may lead to an impairment of EDR was tested in the rabbit model of diabetes. The diabetic state may influence EDR in several ways.

1. It has already been shown that hypercholesterolemia and atherosclerosis influence EDR. Hence the lipid abnormalities associated with diabetes (Ganda, 1985) could influence EDR also.
2. One of the agents postulated to be EDRF is a metabolite of the lipooxygenase pathway of arachidonic acid metabolism. In diabetes, it has been suggested that there are changes in the conversion of linoleic acid to arachidonic acid in different tissues (Huang *et al.*, 1984). Such changes in vascular tissues could influence EDR.
3. One of the primary pathological features in diabetes is seen in the capillary basement membrane. The basement membranes have been shown to be thickened in the microvasculature (Johnson, 1985). It is possible that similar changes could occur in the large blood vessels also. Disease of small blood vessels may intensify disease in larger blood vessels by impairing microvascular blood flow and/or by involvement of vasa vasorum in the large arteries. Such changes could influence both endothelial and smooth muscle function and influence EDR.

The diabetic rabbits of this series had markedly elevated concentrations of serum triglycerides compared to control rabbits. A mild hypercholesterolemia was seen also.

These abnormalities are similar to those described previously (Kloeze and Abdellatif, 1975; Altura *et al.*, 1981). Despite these abnormalities in lipids in the serum, no atherosclerosis was seen in the aortae of diabetic rabbits. The scanning electron microscopic appearances of the aortic endothelium of diabetic rabbits were similar to the appearances of the aortic endothelium in control rabbits. In this study, no impairment in EDR to acetylcholine was seen in the aortae of the diabetic rabbits. These results are similar to those published by Head *et al.*, (1987).

In a study by Oyama *et al* (1986), the EDR to acetylcholine and histamine were impaired in the aortae of Wistar rats made diabetic with streptozotocin (50 mg/kg, i.v.). No endothelial abnormalities were seen at scanning electron microscopy of the intima of these aortae. On the contrary, Head *et al.*, (1987) found no impairment in the EDR to acetylcholine in the aortae of Sprague-Dawley rats made diabetic with streptozotocin (65 mg/kg, i.p.). Except for the strain of rats, the dose and route of administration of streptozotocin, the above two studies had many similarities, especially the duration of diabetes and the range of blood glucose concentrations achieved. The reasons for the differences in results (i.e., impaired EDR to acetylcholine in the former study and lack of impairment of EDR in the latter) between these two studies are unclear.

In a study using the aortae from genetically diabetic biobreeding rats (BB rats), the present investigators found the EDR to actylcholine and histamine impaired in comparison with aortae from non-diabetic controls (Meraji *et al.*, 1987). However, in the diabetic animals of this variety endothelial cell abnormalities were detected by scanning electron microscopy. These appearances included broken cell surfaces and appearance of holes on cells. These structural abnormalities were thought to be responsible for the functional defect leading to the impaired EDR. As endothelial cell abnormalities were seen only in the aortae of genetically diabetic BB rats and not in the aortae of rats and rabbits with chemical diabetes, it appears that the endothelial cell abnormalities in the BB rat are probably related to genetic factors and not to the hyperglycemia.

The endothelial function of the diabetic rabbits of the present study can be influenced by two factors.

1. The state of diabetes *per se* could influence endothelium function.
2. The state of diabetes could predispose to the development of atherosclerosis and hence influence endothelial function.

When studied by scanning and tranmission electron microscopy, no abnormalities were seen in the aortic endothelium of the diabetic rabbits of the present study. The appearances of the cells were similar to those of non-diabetic controls. The EDR to acetylcholine was not impaired also. Hence, the state of diabetes *per se* may not have produced any injurious effect on the endothelial cells.

Similarly, changes suggestive of atherosclerosis were not seen in the aortae of the diabetic rabbits. The serum cholesterol estimations in the diabetic rabbits were significantly lower compared to those seen in cholesterol-fed rabbits. In fact, some authors have suggested that in rabbits, the state of diabetes has a protective effect on the endothelium and inhibits atherosclerosis (Duff and McMillan, 1949). The results discussed in this protocol do not agree with the initial hypothesis that diabetes mellitus in rabbits may lead to an impairment of EDR. Instead, these results suggest that in the absence of atherosclerosis, diabetes *per se* does not impair EDR.

Calcium Channel Blockers and EDR

Calcium channel blockers are used in the treatment of Prinzmetal's angina (Pepine *et al*, 1983). It is believed that coronary artery spasm has a prominent pathogenic role in this disease. In addition, calcium channel blockers are used with benefit in the treatment of stable angina pectoris (Jenkins *et al.*, 1981). This symptom is believed to be due to an imbalance in myocardial oxygen supply and demand. Calcium channel blockers may be improving stable angina by augmenting the oxygen supply or reducing the oxygen demand or both. In addition, as it is postulated that loss of EDR is an early marker of atherosclerosis, the effect of calcium channel blockers on EDR was of interest. Based on

the above considerations it was hypothesized that calcium channel blockers will not lead to an impairment of EDR. The findings of Protocol Four confirmed this hypothesis.

The link between Ca^{++} and EDR is highlighted by the following findings.

1. Calcium ionophore A23187 is a potent mediator of EDR (Furchgott *et al.*, 1983).
2. Calcium agonist Bay K 8644 can mediate EDR (Rubanyi *et al.*, 1986).
3. It was initially believed that EDRF is a metabolite of arachidonic acid generated by the action of phospholipase A_2. Calcium ions facilitate the action of this enzyme and hence could increase production of EDRF (Furchgott, 1983).

In order to consolidate the link between Ca^{++} and EDR, several investigators have examined the influence of zero calcium buffers and calcium channel blockers upon this phenomenon. For instance, Singer and Peach (1982) showed that extracellular calcium depletion inhibited the methacholine induced relaxation. They also showed that the calcium channel blockers nifedipine and verapamil inhibited methacholine and A23187 induced relaxations. Similarly, Long and Stone (1985) using a bioassay system have shown that the acetylcholine evoked release and probably also the basal release of EDRF is dependent upon the presence of extracellular Ca^{++}. Other investigators have failed to abolish the EDR to acetylcholine with the calcium channel blockers verapamil and nifedipine (Winquist *et al.*, 1985).

In the experiments described in the present study, the calcium channel blockers nicardipine and diltiazem had no inhibitory effect on the EDR to acetylcholine. This could be due to one of several factors.

1. Nicardipine and diltiazem may not have calcium channel blocking properties in the rabbit aorta.

This explanation is unlikely as both nicardipine and diltiazem inhibited the potassium induced contraction under the present experimental conditions. Also, nicardipine had been previously shown to inhibit $^{45}Ca^{++}$ uptake and the contractile response induced by high extracellular potassium concentrations in the rabbit aorta (Terai *et*

al., 1981). Similar studies with diltiazem support its action as a calcium channel blocker in the rabbit aorta (Ito *et al.*, 1978; Van Breemen *et al.*, 1981). It must be remembered that the above studies refer to calcium channels in vascular smooth muscle. The assumption that these compounds may exert a similar calcium channel blocking action on the cell membranes of the endothelial cells remains to be proven.

2. Calcium ions are not involved in the EDR to acetylcholine in the rabbit aorta.

This appears unlikely as the EDR to acetylcholine has been shown to be inhibited by exposing the tissue to zero Ca^{++} buffer *in vitro* (Singer and Peach, 1982). In a recent study Collins *et al.*, (1986) monitored the changes in tension and $^{45}Ca^{++}$ movements in the rabbit aorta during relaxations mediated by EDRF, sodium nitroprusside and 8-bromo-cyclic guanosine monophosphate (8-bromo cGMP). During EDR by acetylcholine the noradrenaline induced increase in calcium influx was reduced. Sodium nitroprusside and 8-bromo-cGMP had similar effects on deendothelialized preparations. EDRF also inhibited noradrenaline induced calcium efflux. Sodium nitroprusside and 8-bromo-cGMP had similar effects in deendothelialized rings both in the presence and absence of extracellular calcium. Hence, it was concluded that the vascular smooth muscle relaxant effect of EDRF and nitro vasodilators may be produced by a cyclic-GMP mediated reduction of cytosolic calcium, through both inhibition of calcium influx and reduction of intracellular calcium release. Further, the induction of EDR by the calcium ionophore A23187 and the calcium agonist Bay K 8644, make the proposition that calcium ions are not involved in EDR to acetylcholine an unlikely one (Rubanyi *et al.*, 1986).

3. Calcium translocation during release of EDRF is not blocked by nicardipine and diltiazem.

Although both these drugs have been shown to inhibit Ca^{++} influx induced by high extracellular potassium in the rabbit aorta, these drugs (as well as other calcium channel blockers), do not block all calcium channels. It is generally believed that there are at least three types of calcium channels in the sarcolemma: (1) voltage operated channels which

allow influx of calcium when the cell membrane is depolarized, (2) receptor operated channels which allow calcium influx following occupation of receptors by agonists without a necessary change in the membrane potential and (3) channels mediating calcium influx in the resting state. (Weiss, 1981).

Of these channels, depending on the vascular bed and animal species, the calcium channel blockers could act predominantly on voltage operated channels or receptor operated channels (Cauvin *et al.*, 1983). The Ca^{++} influx induced by depolarization of most cell membranes with high extracellular potassium is inhibited by these drugs (Vanhoutte, 1981). However, the contractile responses produced by exogenous noradrenaline are often resistant to the action of calcium channel blockers (Vanhoutte and Rimele, 1982; Jayakody *et al.*, 1986). Thus, it is possible that any Ca^{++} influx associated with EDR to acetylcholine in the rabbit aorta may utilize calcium channels not blocked by these drugs.

Findings similar to this study have been reported by Winquist *et al.* (1985) in rat and rabbit aortae, where endothelium-dependent relaxations were not altered appreciably by verapamil (10 µmol/l) and only affected modestly by a single concentration of nifedipine (0.5 µmol/l). These authors concluded that although the presence of extracellular calcium is required critically for the expression of EDR, the associated calcium translocation is not blocked by organic calcium channel blockers.

The findings of the present study suggest that the calcium channels which are blocked by nicardipine (which is a dihydropyridine compound) do not appear to be involved in the production of EDRF by acetylcholine as well as the relaxant responses mediated by EDRF in the rabbit aorta. In addition, diltiazem (which is a benzothiazepine compound) is also without effect on EDR. Finally, verapamil (which is a phenylalkylamine compound) has been shown to have no effect on release of EDRF in bioassay experiments (Vanhoutte, 1987b). As drugs from three structurally different classes of calcium channel blockers have shown no significant inhibitory effect on EDR to

acetylcholine in the rabbit aorta it is concluded that the calcium translocation which is believed to occur during (1) production of EDRF by acetylcholine (2) relaxation produced by EDRF is not blocked by organic calcium channel blockers.

Similar conclusions have been arrived at by Vanhoutte (1987b). He concludes that the release of EDRF evoked by acetylcholine is mediated by muscarinic receptors and appears to be a Ca^{++}activated process. However, it is not due to activation of calcium channels sensitive to verapamil or dihydropyridines, although activation of these channels and calcium ionophores can evoke release of EDRF.

Vasospasm and calcium channel blockers

'Vasospasm' can be defined as an abnormal or exaggerated constriction of the blood vessel wall (Vanhoutte, 1978; Van Neuten and Vanhoutte, 1981). It can occur as a generalized or a localized phenomenon. Since contraction of vascular smooth muscle depends on an increase of cytoplasmic calcium, occurrence of spasm must be related to calcium handling by the cell. During vascular smooth muscle contraction, both entry of extracellular calcium and release of calcium from intracellular stores are known to occur. Different blood vessels appear to depend on the external source of activator calcium to different degrees during contractions.

The clinical entity `variant angina' was thought to have a basis of underlying coronary artery spasm (Prinzmetal *et al.*, 1959). Clinical studies suggest that coronary vasospasm is relatively frequent and can be superimposed on partial occlusion of the coronary arteries due to atherosclerosis (Maseri *et al.*, 1979).

High extracellular potassium induced contractions in coronary vascular smooth muscle, which is due to influx of extracellular calcium are inhibited by calcium channel blockers (Van Neuten and Vanhoutte, 1980). Lidoflazine (a calcium channel blocker) has been shown to inhibit contractions of isolated coronary arteries by noradrenaline, serotonin, ergonovine maleate and anoxia (Van Neuten *et al.*, 1980). These experiments on isolated coronary arteries suggest that calcium channel blockers are beneficial in the

treatment of coronary vasospasm. Now it is the accepted practice to treat cardiovascular pathology caused by myocardial ischaemia and coronary vasospasm with calcium channel blockers. In the light of the above practical significance, it is reassuring to note that calcium channel blockers had no significant inhibitory effect upon EDR to acetylcholine. Thus, calcium channel blockers may not be influencing vasospasm through effects on EDR.

Conclusions

The findings and conclusions of the present investigation can be summarized as follows

1. Feeding a diet containing 2% cholesterol and lipids to rabbits leads to the development of atherosclerosis. In this model of experimental atherosclerosis, structural abnormalities in the aortic endothelium were associated with an impairment in EDR to acetylcholine in the aorta.
2. The impairment in EDR in experimental cholesterol atherosclerosis was shown to be due to an impairment in the production of EDRF.
3. When the animals were put back on standard rabbit diet after an initial 6 weeks of feeding the 2% cholesterol diet, no regression of atherosclerosis was seen in the aortae When studied 36 weeks after reversal of diet, an increased deposition of lipids was seen in the aortae. In addition, the EDR to acetylcholine was found to be persistently impaired.
4. When the atherogenic diet was given to young and older rabbits, a dissociation in the extent of atherosclerosis was seen between the two animal groups. The young rabbits showed features of classical atherosclerosis and the EDR to acetylcholine was found to be impaired in the aortae. In the older rabbits, features of classical atherosclerosis were not seen in the aortae. Only electron microscopic evidence of endothelial cell damage was seen. However, the EDR to acetylcholine was found to be impaired. This result suggests that impairment of EDR could be an early marker of atherosclerosis.

5. The EDR to acetylcholine was found to be preserved in aortae of rabbits made diabetic with alloxan. Hence diabetes mellitus *per se*, in the absence of atherosclerosis does not lead to an impairment in EDR.

6. The calcium channel blockers, nicardipine and diltiazem, were found to have no inhibitory effect on EDR to acetylcholine in the rabbit aorta. The beneficial effect of calcium channel blockers in coronary vasospasm may be mediated through mechanisms independent of EDR.

Future directions

The present investigation has demonstrated that feeding a diet containing 2% cholesterol and lipids to rabbits leads to the development of atherosclerosis. In this model of atherosclerosis, structural abnormalities in the endothelium were associated with impairment in EDR to acetylcholine in the aorta. Evidence was also presented suggesting that impairment in EDR is an early marker of atherosclerosis.

The nature and extent of the early changes in the endothelial cells which lead to impaired production of EDRF are unknown. These changes in the endothelial cells may occur gradually over several weeks or occur abruptly over 1-2 days. More basic questions like: is the impairment of EDR in some way related to a particular dose of ingested cholesterol or to a particular serum concentration of cholesterol ? can be raised. Alternatively, the impairment of EDR may not be related to the concentration of serum cholesterol at all but related to the concentration of aortic tissue cholesterol. In order to investigate these possibilities a series of experiments, relating the amounts of ingested cholesterol, serum cholesterol, aortic tissue cholesterol, EDR and ultrastructure need to be performed. In the atherosclerotic rabbits of the present study, a considerable variation in the extent of atherosclerosis was seen among the animals. An important reason for this was the variation in the intake of food. For future studies, this aspect has to be controlled.

In the present study, no regression of atherosclerosis was seen in the rabbits. A scrutiny of the available literature on this subject (regression of atherosclerosis in rabbits),

suggests that when a sufficient concentration of cholesterol is given for a sufficient duration, the atherosclerosis in the rabbit may not be reversible. However, few authors have shown regression of atherosclerosis in the rabbit also (Bortz, 1968). According to Bortz, in the rabbit model of experimental cholesterol atherosclerosis, there is an early reversible phase before fibrous tissue reaction is severe, where reversal of diets will lead to regression of atherosclerosis. In the present experiments, the 2% cholesterol diet was given to the rabbits for 6 weeks. It may be possible that, by feeding a diet containing a lesser concentration of cholesterol for a shorter period of time, a reproducible model showing regression of atherosclerosis can be developed. With such a model, one can ascertain whether there is restoration of EDR with regression of atherosclerosis. This possibility has been shown to be true in the monkey model (Freiman *et al.*, 1986). The effects of different agents like oils obtained from marine fish (fish oils) and calcium channel blockers can also be tested on this model showing regression of atherosclerosis.

There has been considerable interest in the potential role of dietary fatty acids in the prevention of atherosclerosis (Carroll, 1986). These fatty acids (derived from fish oils) not only lower the blood cholesterol but also influence the proportions of unsaturated fatty acids in the membranes of cells. It has been argued that alteration in membrane fluidity may underlie the development of atherosclerosis (Jackson and Gotto, 1976). It would be interesting to examine the ability of dietary supplements of unsaturated fatty acids in fish oils to protect against the atherogenic effect of a high cholesterol diet.

Several studies have shown that the atherogenic effect of cholesterol feeding can be attenuated by the administration of calcium channel blockers (Whittington-Coleman and Carrier, 1970; Henry and Bentley, 1981; Parmley *et al.*, 1985). These studies have been criticized on the grounds that the doses of the drugs used were greatly in excess of those employed in the treatment of clinical syndromes associated with atherosclerosis and vascular spasm in humans. However, if the loss of EDR precedes the development of classical atherosclerosis (as shown in the present study), it would be important to establish

whether these drugs protected against the loss of EDR produced by cholesterol feeding. It is conceivable that the dose of calcium channel blocker required for this purpose could be smaller and hence, in a clinically relevant range.

The effect of different components of the diet on the extent of atherosclerosis seen in rabbits remains unclear. In some studies, no significant correlation had been noted between the amount of cholesterol in the diet and the severity of induced atherosclerosis (Scebat *et al.*, 1964). Lambert *et al.*, (1958) showed that cholesterol was not needed at all, but that diets high in fatty acids, particularly the saturated fatty acids by themselves will induce atherosclerosis. The atherogenic diet used in the present study had 2% cholesterol and was high in saturated fats. The effects of the cholesterol component *per se* and the fatty acid component *per se* on the extent of atherosclerosis and EDR would be interesting to investigate. Also, the compositions of the fatty acids in the diet can be changed (e.g., high in polyunsaturated fatty acids) and their effects on atherosclerosis and EDR studied (Cherry *et al.*,1983). Some of the experimental protocols described in this thesis have been already repeated using a diet supplemented only with 2% cholesterol. These experiments have revealed results similar to those described in the thesis.

The protocol where the effect of age on the susceptibility of rabbits to atherosclerosis was investigated (Protocol Three), was confounded by the persistent loss of weight in these animals. The loss of weight in these rabbits was thought to be due to the poor palatability of the high cholesterol, high fat diet. The palatability of the diet may be improved by reducing the lipid content of the diet. This protocol should be repeated (without loss of weight in animals) while controlling the amount of food ingested.

Andrews *et al.*, (1987) have shown that continuous exposure of the endothelium to high concentrations of LDL leads to impairment of EDR in the normal rabbit aorta. Incubation of normal aortic rings with LDL inhibited EDR to acetylcholine and A23187. As chemical modification of LDL did not influence the relaxation to acetylcholine, the authors concluded that the inhibition of EDR is mediated through a LDL receptor dependent

mechanism. In the rabbit model of atherosclerosis, an abnormal lipoprotein profile is found (Shore *et al.*, 1974; Rodriguez *et al.*, 1976). After a 1% cholesterol diet the VLDL and LDL fractions were found increased and the HDL decreased. The VLDL particles were larger in size and had elevated cholesterol esters and decreased glycerides compared to VLDL of control rabbits. The arginine-rich glycoprotein was greatly increased. The hypercholesterolemic VLDL also contained a protein that may correspond in part at least to the β proteins of human VLDL (Shore *et al.*, 1974). These changes in the serum lipid profile of cholesterol-fed rabbits may influence EDR. As different lipoproteins have been shown to have proatherogenic and antiatherogenic properties, the separate influence of these fractions of lipoproteins on EDR would be interesting.

Finally, investigations may be directed at identifying EDRF. Recent reports suggest that this substance is nitric oxide (Palmer *et al.*, 1987). There is no definitive information about the source of the nitric oxide radical in the cell. Indeed, Vanhoutte (1987 c) has suggested that the nitric oxide radical may be one of a series of such labile factors which are produced within endothelial cells. It would be interesting to investigate the generation of these radicals in the endothelial cells and the effects of cholesterol feeding on these cellular processes. Methods similar to those described by Palmer *et al.* (1987), where they determined the nitric oxide as the chemiluminescent product of its reaction with ozone, could be used for this type of investigation.

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APPENDIX 1

The ingredients used in the formulation of the 2% cholesterol diet were as follows:

Casein	21.00%
Sucrose	15.00%
Solka Floc	13.00%
Vitamin Mix	2.00%
Mineral Mix	5.00%
DL Methionine	0.15%
Choline	0.20%
Lard	20.00%
Dextrin	21.65%
Cholesterol	2.00%

The ingredients used in the formulation of the standard rabbit diet were as follows:

Dehydrated alfalfa

Wheat

Barley

Soymeal

Mill-by-product

Oats

Limestone

Whey powder

Methionine

Choline chloride

Salt

Minerals

Vitamins

APPENDIX 2

Details of preparation of solutions used in METHODS section.

The Millonig's buffer used for TEM studies was prepared in the following manner: 500 ml water was placed in a liter beaker with a large magnetic stirrer, and another 500 ml of water was measured in a cylinder. Amounts of 16.8 g $NaH_2PO_4.H_20$, 3.85 g NaOH and 5.4 g glucose were weighed in separate cups and dissolved in the water in the beaker. Then, 300 ml of water from the cylinder was added to the beaker and mixed. Calcium chloride ($CaCl_2$) 0.05 g was dissolved in 3-5 ml of water in a small beaker. With a Pasteur pipette, the $CaCl_2$ 'solution' was added drop-wise to the chemicals in the beaker. The remaining 200 ml of water in the cylinder was also added to the beaker and the buffer solution was kept at a pH of 7.25 - 7.40 and stored in a refrigerator after mixing thoroughly. The water used was double distilled for electron microscopy work.

The uranyl acetate solution was prepared in the following way. Uranyl acetate was added to a dark bottle containing 100 ml of water. Once the solution was totally saturated, 100 ml of absolute alcohol was added and mixed. This solution was allowed to settle for 3-4 days and was stored at room temperature.

The lead citrate solution was prepared by dissolving 3.52 g of sodium citrate ($Na_2C_6H_5O_7.2H_2O$) in 80 ml of water. To this solution 2.66 g of lead citrate was added. A white flocculation formed. This 'solution' was stirred with a magnetic stirrer for 30 minutes and then, 20 ml of 1 M NaOH was added to remove the flocculation. This solution was allowed to settle for 4-5 days and was stored at room temperature.

The Sudan Red solution was prepared by mixing 5.0 g of Sudan Red with 500 ml 70% ethyl alcohol and 500 ml of acetone. This solution was filtered three times before use.

APPENDIX 3

Drugs and chemicals used in the study and their sources are given below.

1. Acetylcholine chloride: Mol. Wt. 181.7, Sigma Chemical Co., St. Louis, USA.
2. Alloxan monohydrate: Formula Wt. 160.1, Sigma Chemical Co., St. Louis, USA.
3. Ascorbic acid: Mol. Wt. 176.1, Sigma Chemical Co., St. Louis, USA.
4. Atropine: Mol. Wt. 289.4, Sigma Chemical Co., St. Louis, USA.
5. Calcium ionophore A23187: Mol. Wt. 523.6, Sigma Chemical Co., St. Louis, USA.
6. Carbamylcholine chloride: Mol. Wt. 182.6, Sigma Chemical Co., St. Louis, USA.
7. Clonidine Hydrochloride: Mol. Wt. 266.6, Sigma Chemical Co., St. Louis, USA.
8. Diltiazem hydrochloride: Mol. Wt. 451.0, Sigma Chemical Co., St. Louis, USA.
9. Dimethylsulfoxide (DMSO): Mol. Wt. 84.14, Sigma Chemical Co., St. Louis, USA.
10. Disodium calcium ethylenediaminetetraacetic acid: Mol. Wt. 374.3, Sigma Chemical Co., St. Louis, USA.
11. Hydroquinone: Mol. Wt. 110.1, Sigma Chemical Co., St. Louis, USA.
12. Indomethacin: Mol. Wt. 357.8, Sigma Chemical Co., St. Louis, USA.
13. Methacholine chloride: Mol. Wt. 195.7, Sigma Chemical Co., St. Louis, USA.
14. Methoxamine hydrochloride: Mol. Wt. 247.7, Burroughs Wellcome Ltd., Montreal, Canada.
15. Nicardipine hydrochloride: Mol. Wt. 516.04, Syntex Research, Palo Alto, CA., USA.
16. Noradrenaline bitartarate: Mol. Wt. 319.3, Sigma Chemical Co., St. Louis, USA.
17. Nordihydroguaiaretic acid (NDGA): Mol. Wt. 302.4, Sigma Chemical Co., St. Louis, USA.
18. Pentobarbitone sodium (Somnotol): M.T.C. Pharmaceuticals, Mississauga, Ontario, Canada.
19. Propranolol hydrochloride: Mol. Wt. 295.8, Sigma Chemical Co., St. Louis, USA.
20. Prostaglandin $F_{2\alpha}$ (Prostin $F_{2\alpha}$, Dinoprost tromethamine): Mol. Wt. 475.6, Upjohn Company of Canada, Don Mills, Ontario, Canada.

21. Quinacrine hydrochloride: Mol. Wt. 472.88, Sigma Chemical Co., St. Louis, USA

22. Sodium nitrite: Mol. Wt. 69.00, Sigma Chemical Co., St. Louis, USA.

The concentrated stock solutions (-4.0 log mol/l in most cases) of the drugs were prepared in distilled water. Sodium metabisulphite (1.0 mg) was added to each 10 ml of the stock solutions to minimize oxidation in the case of easily oxidisable drugs. Suitable dilutions of the drugs were prepared in Krebs-bicarbonate buffer solution each day.

Indomethacin was dissolved in an equimolar Na_2C0_3 solution. NDGA and A23187 were dissolved in dimethylsulfoxide (DMSO). Subsequent dilutions were made in distilled water. The final bath concentration of DMSO did not exceed 0.025 mol%. DMSO in this concentration had no effect on the rabbit aorta, on the contractile response to noradrenaline or on the relaxation to acetylcholine. All drugs were added in 20-146 µl aliquots to the tissue bath to produce the desired concentration in the bath fluid. All concentrations are expressed as the final concentration in the tissue bath fluid.